A Cleavable Linker Based on the Levulinoyl Ester for Activity-Based Protein Profiling

Geurink, Paul P.; Florea, Bogdan I.; Li, Nan; Witte, Martin D.; Verasdonck, Joeri; Kuo, Chi-Lin; Marel, Gijs A. van der; Overkleeft, Herman S.

Published in:
Angewandte Chemie International Edition

DOI:
10.1002/anie.201001767

Publication date:
2010

Angewandte Chemie
Eine Zeitschrift der Gesellschaft Deutscher Chemiker

Supporting Information

69451 Weinheim, Germany

A Cleavable Linker Based on the Levulinoyl Ester for Activity-Based Protein Profiling**

*Paul P. Geurink, Bogdan I. Florea, Nan Li, Martin D. Witte, Joeri Verasdonck, Chi-Lin Kuo, Gijs A. van der Marel, and Herman S. Overkleeft**

anie_201001767_sm_miscellaneous_information.pdf

Contents:

General methods

Tetrahydrofuran was distilled over $LiAlH_4$ prior to use. Acetonitrile (ACN), dichloromethane (DCM), *N,N*-dimethylformamide (DMF), methanol (MeOH), diisopropylethylamine (DiPEA) and trifluoroacetic acid (TFA) were of peptide synthesis grade, purchased at Biosolve, and used as received. All general chemicals (Fluka, Acros, Merck, Aldrich, Sigma) were used as received. *O*-(1H-6-Chlorobenzotriazolyl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HCTU) was purchased at Iris Biotech (Marktrewitz, Germany). Traces of water were removed from reagents used in reactions that require anhydrous conditions by co-evaporation with toluene. Solvents that were used in reactions were stored over 4 Å molecular sieves, except methanol and acetonitrile which were stored over 3 Å molecular sieves. Molecular sieves were flame dried before use. Unless noted otherwise all reactions were performed under an argon atmosphere. Column chromatography was performed on Screening Devices b.v. Silica Gel, with a particle size of 40-63 μm and pore diameter of 60 Å. The eluents toluene, ethyl acetate (EtOAc) and petroleum ether (PE) (40-60 °C boiling range) were distilled prior to use. TLC analysis was conducted on Merck aluminium sheets (Silica gel 60 F_{254}). Compounds were visualized by UV absorption (254 nm), by spraying with a solution of $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ (25 g/L) and $(NH_4)_4Ce(SO_4)_4\cdot 2H_2O$ (10 g/L) in 10% sulfuric acid, a solution of $KMnO_4$ (20 g/L) and K_2CO_3 (10 g/L) in water, or ninhydrin (0.75 g/L) and acetic acid (12.5 mL/L) in ethanol, where appropriate, followed by charring at ca. 150 °C. ^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker AV-400 (400 MHz) spectrometer. Chemical shifts are given in ppm (δ) relative to tetramethylsilane, CD_3OD or $CDCl_3$ as internal standard. High resolution mass spectra were recorded by direct injection (2 μL of a 2 μM solution in water/acetonitrile 50/50 (v/v) and 0.1% formic acid) on a mass spectrometer (Thermo Finnigan LTQ Orbitrap) equipped with an electrospray ion source in positive mode (source voltage 3.5 kV, sheath gas flow 10, capillary temperature 250 °C) with resolution R = 60,000 at *m/z* 400 (mass range *m/z* = 150-2000) and dioctylpthalate (*m/z* = 391.28428) as a "lock mass". The high resolution mass spectrometer was calibrated prior to measurements with a calibration mixture (Thermo Finnigan). Optical rotations $[\alpha]_D^{23}$ were recorded on a Propol automatic polarimeter. LC-MS analysis was performed on a Jasco HPLC system with a Phenomenex Gemini 3 lm C18 50 × 4.60 mm column (detection simultaneously at 214 and 254 nm), coupled to a PE Sciex API 165 mass spectrometer with ESI (System A) or a Finnigan Surveyor HPLC system with a Gemini C18 50 × 4.60 mm column (detection at 200-600 nm), coupled to a Finnigan LCQ Advantage Max mass spectrometer with ESI (System B). The applied buffers were H_2O, ACN and 1.0% aq. TFA. Unless noted otherwise the gradient used was 10% → 90% ACN/0.1% aq. TFA. HPLC purifications were performed on a Gilson HPLC system coupled to a Phenomenex Gemini 5 μm 250 × 10 mm column and a GX281 fraction collector. The applied buffers were: 0.1% aq. TFA and ACN. Appropriate fractions were pooled, and concentrated in a Christ rotary vacuum concentrator overnight at room temperature at 0.1 mbar.

Compounds

4-hydroxy-3,5-diisopropylbenzaldehyde (3)[1]

2,6-diisopropylphenol (**2**, 18.4 g, 100 mmol) was dissolved in AcOH (83 mL) and H_2O (17 mL). To this was added hexamine (2 eq., 200 mmol, 28.0 g) and the mixture was heated to reflux for 5 min. after which a distillation head was installed and ca. 9 mL distillate was collected at 110 ºC. The distillation head was removed again and the mixture was refluxed for another 2.5 h after which TLC analysis indicated complete consumption of the phenol starting compound. Next, the mixture was cooled to RT and H_2O (20 mL) was added. Upon further cooling to 0 ºC a pale yellow solid precipitated. The mixture was allowed to stand at 0 ºC for 1 h followed by filtration of the solid. The residue was washed two times with ice-cold water and dried at 60 ºC under reduced pressure. The title compound was obtained without further purification as a pale yellow solid (yield: 20.1 g, 97.6 mmol, 97%). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 9.79 (s, 1H), 7.62 (s, 2H), 4.14 (bs, 1H), 3.30 (p, J = 6.80 Hz, 2H), 1.28 (d, J = 6.80 Hz, 12H). ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm 192.35, 157.16, 135.26, 128.67, 126.01, 26.47, 22.24.

4-(benzyloxy)-3,5-diisopropylbenzaldehyde (4)

Phenol **3** (4.14 g, 20.0 mmol) was dissolved in acetone (100 mL) and to this were added benzylbromide (1.01 eq., 20.2 mmol, 3.46 g) and K_2CO_3 (2 eq., 40.0 mmol, 5.53 g). The suspension was stirred vigorously for 14 h after which TLC analysis revealed a completed reaction. The mixture was concentrated under reduced pressure, redissolved in EtOAc (100 mL) and extracted with H_2O and brine. After drying ($MgSO_4$) and concentration *in vacuo* of the organic layer the title compound was obtained as a colorless oil (yield: 5.50 g, 18.6 mmol, 93%). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 9.95 (s, 1H), 7.70 (s, 2H), 7.48 (d, J = 7.18 Hz, 2H), 7.41 (t, J = 7.30 Hz, 2H), 7.35 (t, J = 7.20 Hz, 1H), 4.86 (s, 2H), 3.41 (sept., J = 6.85 Hz, 2H), 1.27 (d, J = 6.96 Hz, 12H). ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm 191.68, 158.53, 143.13, 136.76, 133.13, 128.50, 128.07, 127.25, 126.13, 76.42, 26.66, 23.74.

(*E*)-*tert*-butyl 3-(4-(benzyloxy)-3,5-diisopropylphenyl)acrylate (6)

To a stirred solution of aldehyde **4** (0.23 g, 0.76 mmol) and *tert*-butyl 2-(diethoxyphosphoryl)acetate (**5**, 1.5 eq., 1.14 mmol, 0.29 g) in THF (10 mL) at 0 ºC was added NaH (1.5 eq., 1.14 mmol, 46.0 mg). The reaction mixture was stirred for 1 h at RT after which TLC analysis indicated a completed reaction. EtOAc (10 mL) was added and the mixture was extracted with 0.1 M aq. HCl (2×), sat. aq. $NaHCO_3$ and brine. The organic layer was dried over $MgSO_4$ and concentrated under reduced pressure. The title compound was obtained after purification by column chromatography (100% PE → 5% EtOAc/PE) as a colorless oil (yield: 0.31 g, 0.77 mmol, quant.). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 7.60 (d, J = 15.94 Hz, 1H), 7.47 (d, J = 7.08 Hz, 2H), 7.40 (t, J = 7.31 Hz, 2H), 7.33 (t, J = 7.24 Hz, 1H), 7.30 (s, 2H), 6.33 (d, J = 15.93 Hz, 1H), 4.81 (s, 2H), 3.38 (sept., J = 6.89 Hz, 2H), 1.54 (s, 9H), 1.24 (d, J = 6.91 Hz, 12H). ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm 166.38, 154.87, 143.78, 142.42, 137.20, 130.97, 128.47, 127.94, 127.26, 124.02, 118.73, 80.14, 76.36, 28.13, 26.54, 23.88. HRMS: calcd. for $C_{26}H_{34}O_3$ 395.25807 $[M+ H]^+$; found 395.25797.

***Tert*-butyl 3-(4-hydroxy-3,5-diisopropylphenyl)propanoate (7)**

Compound **6** (0.30 g, 0.76 mmol) was dissolved in MeOH (10 mL) and the solution was bubbled through with argon for 15 min. before Pd/C 10% w/w (10 mg) was added. The flask was charged with hydrogen for 1 h, after which TLC analysis indicated complete reduction. Argon was bubbled through for another 15 min. and all solids were removed by filtration over Celite. The title compound was obtained after evaporation of the solvent under reduced pressure as a colorless oil (yield: 0.22 g, 0.71 mmol, 94%). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 6.87 (s, 2H), 5.09 (s, 1H), 3.17 (sept., J = 6.80 Hz, 2H), 2.85 (t, J = 7.84 Hz, 2H), 2.52 (t, J = 7.86 Hz, 2H), 1.42 (s, 9H), 1.24 (d, J = 6.91 Hz, 12H). ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm 172.73, 148.26, 133.75, 132.36, 123.10, 80.17, 37.51, 30.83, 27.98, 27.00, 22.73. HRMS: calcd. for $C_{19}H_{30}O_3$ 329.20872 $[M+ Na]^+$; found 329.20871.

Diethyl 3,3'-(1,3-dioxolane-2,2-diyl)dipropanoate (9)[2]

Diethyl-4-oxopimelate (**8**, 11.4 g, 48.4 mmol), ethylene glycol (436 mmol, 24.0 mL) and PPTS (7.26 mmol, 1.82 g) were dissolved in toluene (50 mL) and the mixture was heated to reflux under Dean-Stark conditions for 2 h, after which TLC analysis indicated complete consumption of the ketone. The mixture was cooled to RT and extracted twice with sat. aq. $NaHCO_3$ (50 mL). The organic layer was dried over $MgSO_4$ and concentrated under reduced pressure. The title compound was obtained as a colorless liquid (yield: 13.2 g, 48.4 mmol, quant.). ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 4.13 (q, J = 7.13 Hz, 4H), 3.94 (s, 4H), 2.37 (t, J = 7.65 Hz, 4H), 1.98 (t, J = 7.64 Hz, 4H), 1.25 (t, J = 7.14 Hz, 6H). ^{13}C NMR (75.5 MHz, $CDCl_3$) δ ppm 173.24, 109.84, 65.02, 60.20, 32.07, 28.78, 14.07.

Ethyl 3-(2-(3-hydroxypropyl)-1,3-dioxolan-2-yl)propanoate (10)

A solution of KOH (47.7 mmol, 47.7 mL; 1 M in EtOH) was added dropwise to diethyl ester **9** (13.2 g, 47.7 mmol) at 50 ºC in 4 h. The resulting mixture was stirred at 50 ºC for 14 h after which all EtOH was evaporated under reduced pressure. The resulting residue was suspended in THF (250 mL) and Et_3N (0.5 eq., 23.8 mmol, 3.31 mL) and ethyl chloroformate (1.5 eq., 71.5 mmol, 6.84 mL) were added. After vigorously stirring for 2 h the mixture was added to a cooled (0 ºC) solution of $NaBH_4$ (1.5 eq., 71.5 mmol, 2.71 g) in H_2O (250 mL) and the mixture was stirred at RT for 1 h. The reaction was quenched by addition of 1 mM aq. HCl (100 mL) and the resulting mixture was extracted with Et_2O (3×). The combined organic layers were extracted with brine, dried over $MgSO_4$ and concentrated under reduced pressure. The title compound was obtained after purification by column chromatography (25% → 100% EtOAc/PE) as a colorless liquid (yield: 4.14 g, 17.8 mmol, 37%). ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 4.13 (q, J = 7.13 Hz, 2H), 3.96 (s, 4H), 3.64 (t, J = 5.83 Hz, 2H), 2.37 (t, J = 7.75 Hz, 2H), 2.01 (t, J = 7.65 Hz, 2H), 1.93 (bs, 1H), 1.76-1.62 (m, 4H), 1.26 (t, J = 7.13 Hz, 3H). ^{13}C NMR (75.5 MHz, $CDCl_3$) δ ppm 208.84, 110.72, 65.00, 62.84, 60.34, 33.79, 31.90, 28.92, 26.89, 14.15. HRMS: calcd. for $C_{11}H_{20}O_5$ 255.12029 $[M+ Na]^+$; found 255.12036.

Ethyl 3-(2-(3-(tosyloxy)propyl)-1,3-dioxolan-2-yl)propanoate (11)

Alcohol **10** (4.14 g, 17.8 mmol) was dissolved in DCM (125 mL) and Et_3N (2.1 eq., 37.4 mmol, 5.18 mL), DMAP (0.25 eq., 4.45 mmol, 0.50 g) and TsCl (2.55 eq., 8.66 mmol, 45.4 g) were added. The mixture was stirred for 4 h after which TLC analysis indicated a complete consumption of the starting compound. DCM was

evaporated under reduced pressure and the residue was dissolved in EtOAc, extracted with 1 mM aq. HCl (2×) and brine, dried over $MgSO_4$ and concentrated under reduced pressure. The title compound was obtained after purification by column chromatography (25% → 50% EtOAc/PE) as a colorless liquid (yield: 5.87 g, 15.2 mmol, 85%). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 7.79 (d, *J* = 8.0 Hz, 2H), 7.34 (d, *J* = 8.4 Hz, 2H), 4.12 (q, *J* = 7.1 Hz, 2H), 4.04 (t, *J* = 6.4 Hz, 2H), 3.9-3.8 (m, 4H), 2.45 (s, 3H), 2.31 (t, *J* = 7.6 Hz, 2H), 1.92 (t, *J* = 7.6 Hz, 2H), 1.8-1.7 (m, 2H), 1.7-1.6 (m, 2H), 1.26 (t, *J* = 6.8 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm 173.38, 144.80, 133.30, 129.80, 127.86, 110.15, 70.53, 65.06, 60.36, 33.01, 32.07, 28.83, 23.39, 21.60, 14.19.

Ethyl 3-(2-(3-azidopropyl)-1,3-dioxolan-2-yl)propanoate (12)

A solution of tosylate **11** (5.87 g, 15.2 mmol) and NaN_3 (1.2 eq., 18.2 mmol, 1.19 g) in DMF (120 mL) was stirred at 75 ºC for 14 h, after which TLC analysis indicated a complete conversion. The mixture was concentrated under reduced pressure and the residue was dissolved in EtOAc, extracted with sat. aq. $NaHCO_3$ (2×), H_2O and brine, dried over $MgSO_4$ and concentrated under reduced pressure. The title compound was obtained as a colorless liquid (yield: 3.86 g, 15.0 mmol, 99%). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 4.13 (q, *J* = 7.14 Hz, 2H), 3.94 (s, 4H), 3.32-3.26 (m, 2H), 2.36 (t, *J* = 7.60 Hz, 2H), 1.99 (t, *J* = 7.60 Hz, 2H), 1.71-1.65 (m, 4H), 1.26 (t, *J* = 7.15 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm 173.23, 110.20, 64.93, 60.17, 51.29, 34.07, 31.94, 28.73, 23.24, 14.04. HRMS: calcd. for $C_{11}H_{19}N_3O_4$ 258.14483 $[M+H]^+$; found 258.14491. IR film (cm^{-1}) 2954.7, 2885.3, 2360.7, 2090.7, 1728.1, 1450.4, 1257.5, 1180.3, 1134.1, 1033.8, 948.9, 910.3, 864.1.

3-(2-(3-azidopropyl)-1,3-dioxolan-2-yl)propanoic acid (13)

Ethyl ester **12** (3.86 g, 15.0 mmol) was dissolved in MeOH (80 mL) and to this was added NaOH (4 eq., 60.0 mmol, 30 mL; 2 M in H_2O) at 0 ºC. The mixture was allowed to slowly warm to RT and was stirred for 14 h after which TLC analysis revealed complete conversion. The mixture was concentrated under reduced pressure and the residue was dissolved in sat. aq. $NaHCO_3/H_2O$ (3:1 v/v, 80 mL) and extracted twice with EtOAc. Next, the aqueous layer was acidified with 10% w/v aq. HCl until pH 2 and extracted again twice with EtOAc. The latter organic layers were combined, dried over $MgSO_4$ and concentrated under reduced pressure, which yielded the title compound as a colorless liquid (yield: 3.05 g, 13.3 mmol, 89%). ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 10.20 (bs, 1H), 3.96 (s, 4H), 3.34-3.25 (m, 2H), 2.41 (t, *J* = 7.50 Hz, 2H), 2.00 (t, *J* = 7.51 Hz, 2H), 1.71-1.65 (m, 4H). ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm 179.31, 110.16, 64.95, 51.23, 34.07, 31.68, 28.46, 23.21.

7-azido-4-oxoheptanoic acid (14)

Concentrated aq. HCl (19 mL) was added to a solution of compound **13** (3.05 g, 13.3 mmol) in THF (60 mL) and the mixture was stirred for 3 h, after which TLC analysis showed complete consumption of starting material. Water (150 mL) was added carefully and the aqueous layer was extracted with EtOAc three times. The combined organic layers were extracted with brine, dried over $MgSO_4$ and concentrated *in vacuo*. The title compound was obtained as a colorless oil (yield: 2.46 g, 13.3 mmol, quant.) without further purification necessary. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 9.26 (bs, 1H), 3.32 (t, *J* = 6.64 Hz, 2H), 2.73 (t, *J* = 6.20 Hz,

2H), 2.64 (t, J = 6.16 Hz, 2H), 2.57 (t, J = 7.06 Hz, 2H), 1.88 (p, J = 6.86 Hz, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm 207.65, 178.28, 50.56, 39.10, 36.80, 27.68, 22.79.

4-(3-(*tert*-butoxy)-3-oxopropyl)-2,6-diisopropylphenyl 7-azido-4-oxoheptanoate (15)

To a solution of alcohol **7** (1 eq., 13.3 mmol, 4.08 g), carboxylic acid **14** (1 eq., 13.3 mmol, 2.46 g) and DMAP (0.1 eq., 1.33 mmol, 0.16 g) in DCM (100 mL) was added DIC (1.2 eq., 16.0 mmol, 2.51 mL) and the mixture was stirred for 14 h. Next, the mixture was concentrated under reduced pressure, the residue dissolved in EtOAc (100 mL), extracted with 1 M aq. HCl (2×), sat. aq. $NaHCO_3$ and brine, dried ($MgSO_4$) and concentrated under reduced pressure. The title compound was obtained after purification by column chromatography (100% Tol → 10% EtOAc/Tol) as a colorless oil (yield: 4.41 g, 9.64 mmol, 73%). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 6.96 (s, 2H), 3.30 (t, J = 6.68 Hz, 2H), 2.93-2.80 (m, 8H), 2.58 (t, J = 7.06 Hz, 2H), 2.53 (t, J = 7.83 Hz, 2H), 1.87 (p, J = 6.88 Hz, 2H), 1.42 (s, 9H), 1.17 (d, J = 6.91 Hz, 12H). ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm 207.21, 172.21, 171.48, 143.67, 140.03, 138.61, 123.69, 80.15, 50.52, 39.19, 36.95, 36.88, 30.90, 27.95, 27.65, 27.32, 22.85. HRMS: calcd. for $C_{26}H_{39}N_3O_5$ 496.27819 $[M+ Na]^+$; found 496.27775.

3-(4-((7-azido-4-oxoheptanoyl)oxy)-3,5-diisopropylphenyl)propanoic acid (16)

TFA (10 mL) was added to a solution of *tert*-butyl ester **15** (1.40 g, 3.06 mmol) in DCM (10 mL) and this mixture was stirred for 30 min. after which TLC analysis showed complete consumption of starting material. Toluene (25 mL) was added and the mixture was concentrated under reduced pressure. In order to remove traces of TFA the mixture was coevaporated with toluene three times. The title compound was obtained as a colorless oil (yield: 1.41 g, 3.52 mmol, quant.). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 9.40 (s, 1H), 6.98 (s, 2H), 3.29 (t, J = 6.69 Hz, 2H), 2.95-2.81 (m, 8H), 2.67 (t, J = 7.91 Hz, 2H), 2.59 (t, J = 7.06 Hz, 2H), 1.87 (p, J = 6.92 Hz, 2H), 1.17 (d, J = 6.91 Hz, 12H). ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm 207.43, 178.29, 171.58, 143.75, 140.18, 138.08, 123.64, 50.44, 39.14, 36.82, 35.59, 30.48, 27.59, 27.27, 22.79. HRMS: calcd. for $C_{22}H_{31}N_3O_5$ 440.21559 $[M+ Na]^+$; found 440.21539.

4-(3-((2,5-dioxopyrrolidin-1-yl)oxy)-3-oxopropyl)-2,6-diisopropylphenyl 7-azido-4-oxoheptanoate (17)

N-hydroxysuccinimide (1.5 eq., 1.30 mmol, 150 mg) and EDC (1.5 eq., 1.30 mmol, 249 mg) were added to a solution of carboxylic acid **16** (0.36 g, 0.87 mmol) in DCM (7 mL) and the mixture was stirred for 14 h, after which TLC analysis indicated complete consumption of starting material. The solvent was evaporated under reduced pressure and the residue was dissolved in EtOAc (20 mL). This was extracted with 1 M aq. HCl (2×) and brine, dried over $MgSO_4$ and concentrated under reduced pressure. The title compound was obtained after purification by column chromatography (30% → 60% EtOAc/PE) as a colorless oil (yield: 0.43 g, 0.83 mmol, 95%). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 6.99 (s, 2H), 3.31 (t, J = 6.67 Hz, 2H), 3.06-3.00 (m, 2H), 2.95-2.81 (m, 12H), 2.60 (t, J = 7.06 Hz, 2H), 1.88 (p, J = 6.90 Hz, 2H), 1.18 (d, J = 6.89 Hz, 12H). ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm 207.26, 171.53, 169.03, 167.90, 144.16, 140.59, 137.15, 123.75, 50.60, 39.28, 36.95, 32.70, 30.35, 27.70, 27.44, 25.54, 22.91. LC-MS (System A): R_t (min): 9.38 (ESI-MS (m/z): 515.4 ($M + H^+$)). HRMS: calcd. for $C_{26}H_{34}N_4O_7$ 515.25003 $[M+ H]^+$; found 515.24963.

Fmoc-Ile-Thr(*t*Bu)-OMe (22)

Fmoc-Ile-OH (1.2 eq., 13.3 mmol, 4.70 g) was dissolved in DCM (60 mL) and to this were added HCTU (1.2 eq., 13.3 mmol, 5.50 g), DiPEA (3.3 eq., 36.0 mmol, 6.0 mL) and HCl·H-Thr(*t*Bu)-OH (1 eq., 11.0 mmol, 2.50 g) successively. The mixture was stirred for 2 h after which TLC analysis indicated a completed reaction. DCM was evaporated under reduced pressure and the residue was dissolved in EtOAc, extracted with 1 M aq. HCl (2×), sat. aq. $NaHCO_3$ (2×) and brine, dried over $MgSO_4$ and concentrated under reduced pressure. The title compound was obtained after purification by column chromatography (10% → 50% EtOAc/PE) as a colorless solid (yield: 5.16 g, 9.83 mmol, 89%). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 7.76 (d, *J* = 7.48 Hz, 2H), 7.60 (d, *J* = 7.41 Hz, 2H), 7.39 (t, *J* = 7.46 Hz, 2H), 7.31 (dt, *J* = 7.43, 0.98 Hz, 2H), 6.48 (d, *J* = 8.84 Hz, 1H), 5.58 (d, *J* = 8.70 Hz, 1H), 4.49 (dd, *J* = 9.00, 1.68 Hz, 1H), 4.44-4.33 (m, 2H), 4.28-4.21 (m, 2H), 4.18 (dd, *J* = 8.53, 6.39 Hz, 1H), 3.71 (s, 3H), 1.94-1.83 (m, 1H), 1.65-1.53 (m, 1H), 1.33-1.21 (m, 1H), 1.17 (d, *J* = 6.27 Hz, 3H), 1.11 (s, 9H), 1.02-0.94 (m, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm 171.42, 170.86, 156.07, 143.78, 141.24, 127.63, 127.01, 125.07, 119.90, 74.21, 67.19, 66.96, 59.30, 57.83, 52.13, 47.17, 38.18, 28.27, 24.82, 21.04, 15.08, 11.52. LC-MS (System B): R_t (min): 10.87 (ESI-MS (m/z): 525.0 ($M + H^+$)).

Boc-Ile-Ile-Thr(*t*Bu)-$NHNH_2$ (23)

DBU (1.05 eq., 10.3 mmol, 1.57 mL) was added to a solution of Fmoc-Ile-Thr(*t*Bu)-OMe (**22**, 5.16 g, 9.83 mmol) in DMF (50 mL) and the mixture was stirred for 5 min. after which HOBt (1.5 eq., 14.7 mmol, 1.98 g) was added. After stirring the mixture for another 30 min. Boc-Ile-OH (1.2 eq., 11.8 mmol, 2.73 g), HCTU (1.2 eq., 11.8 mmol, 4.88 g) and DiPEA (3 eq., 29.5 mmol, 4.87 mL) were added. TLC analysis indicated sufficient product formation after 14 h and the mixture was concentrated under reduced pressure. The residue was redissolved in DCM and extracted with 1 M aq. HCl (2×), sat. aq. $NaHCO_3$ (2×) and brine, dried over $MgSO_4$ and concentrated *in vacuo*. The intermediate was purified by column chromatography (10% → 50% EtOAc/PE) and the obtained product (3.69 g, 7.15 mmol) was dissolved in MeOH (50 mL). Hydrazine monohydrate (30 eq., 215 mmol, 10.4 mL) was added and the mixture was stirred for 14 h, after which TLC analysis indicated complete consumption of starting material. Toluene was added and the mixture was concentrated under reduced pressure. The title compound was obtained after coevaporation with toluene (3×) as a colorless solid (yield: 3.66 g, 7.10 mmol, 72%). ^{1}H NMR (400 MHz, CD_3OD) δ ppm 4.34 (d, *J* = 3.53 Hz, 1H), 4.29 (d, *J* = 8.12 Hz, 1H), 4.05-3.99 (m, 1H), 3.92 (d, *J* = 7.90 Hz, 1H), 1.90-1.70 (m, 2H), 1.61-1.47 (m, 2H), 1.42 (s, 3H), 1.22-1.10 (m, 1H), 1.17 (s, 9H), 1.08 (d, *J* = 6.32 Hz, 3H), 0.92-0.85 (m, 12H). ^{13}C NMR (100 MHz, CD_3OD) δ ppm 174.83, 173.39, 171.30, 157.91, 80.56, 75.84, 68.52, 60.62, 59.22, 58.56, 37.94, 37.85, 28.77, 28.66, 25.94, 19.78, 16.23, 15.95, 11.39, 11.32. LC-MS (System A): R_t (min): 6.08 (ESI-MS (m/z): 516.4 ($M + H^+$)). HRMS: calcd. for $C_{25}H_{49}N_5O_6$ 516.37556 $[M+ H]^+$; found 516.37530.

Boc-Ile-Ile-Thr(*t*Bu)-Leu-EK (24)

Boc-Ile-Ile-Thr(*t*Bu)-$NHNH_2$ (**23**, 1 eq., 3.87 mmol, 2.0 g) was dissolved in a 9:1 v/v mixture of DCM/DMF (40 mL) and cooled to –30 ºC. To this were added *t*BuONO (1.1 eq., 4.25 mmol, 0.57 mL) and HCl (2.8 eq., 10.8 mmol, 2.70 mL; 4 M in 1,4-dioxane). This mixture was stirred at –30 ºC for 3 h, after which (*S*)-2-amino-4-methyl-1-((*R*)-2-methyloxiran-2-yl)pentan-1-one TFA salt[3] (1.1 eq., 4.25 mmol, 1.16 g) in DMF (5 mL) and

DiPEA (5 eq., 20.0 mmol, 3.31 mL) were added. The reaction was allowed to warm to RT and stirred for 14 h. Next, DCM (15 mL) was added and the mixture was extracted with 0.1 M aq., HCl (2×) and H_2O, dried over $MgSO_4$ and concentrated under reduced pressure. The title compound was obtained after purification by column chromatography (20% → 50% EtOAc/PE) as a colorless solid (yield: 2.25 g, 3.43 mmol, 89%). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 7.64 (d, *J* = 7.47 Hz, 1H), 6.99 (d, *J* = 5.63 Hz, 1H), 6.45 (d, *J* = 8.22 Hz, 1H), 5.22 (d, *J* = 7.84 Hz, 1H), 4.46 (ddd, *J* = 10.45, 7.55, 2.94 Hz, 1H), 4.41-4.32 (m, 2H), 4.14-4.07 (m, 1H), 3.94 (t, *J* = 7.34 Hz, 1H), 3.38 (d, *J* = 5.07 Hz, 1H), 2.89 (d, *J* = 5.06 Hz, 1H), 1.92-1.77 (m, 2H), 1.74-1.63 (m, 1H), 1.60-1.55 (m, 1H), 1.54-1.48 (m, 2H), 1.52 (s, 3H), 1.44 (s, 9H), 1.33-1.24 (m, 1H), 1.28 (s, 9H), 1.19-1.08 (m, 2H), 1.06 (d, *J* = 6.44 Hz, 3H), 0.96 (d, *J* = 6.54 Hz, 6H), 0.92-0.86 (m, 12H). ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm 208.06, 171.59, 170.73, 169.51, 155.80, 79.76, 75.49, 66.14, 59.24, 57.68, 56.95, 52.39, 50.74, 39.80, 37.30, 36.97, 28.28, 28.08, 25.42, 24.88, 24.69, 23.35, 21.35, 16.75, 16.33, 15.53, 15.40, 11.28. LC-MS (System B): R_t (min): 11.33 (ESI-MS (m/z): 655.27 ($M + H^+$)). HRMS: calcd. for $C_{34}H_{62}N_4O_8$ 655.46404 $[M+ H]^+$; found 655.46451.

N_3-Lev-phenol-epoxomicin (19)

Compound **24** (165 mg, 0.25 mmol) was treated with a 1:1 v/v mixture of DCM/TFA (2 mL) for 1 h and subsequently coevaporated with toluene (3×). The resulting intermediate was dissolved in DMF (2 mL) and to this were added compound **17** (1.1 eq., 0.258 mmol, 144 mg) and DiPEA (2 eq., 0.50 mmol, 83 µL). The reaction was stirred for 14 h before being concentrated under reduced pressure. The residue was dissolved in DCM (10 mL) and extracted with 1 M aq. HCl (2×), sat. aq. $NaHCO_3$ (2×) and brine, dried over $MgSO_4$ and concentrated under reduced pressure. The title compound was obtained after purification by column chromatography (25% → 100% EtOAc/PE) as a colorless solid (yield: 141 mg, 0.16 mmol, 63%). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 8.75 (s, 1H), 8.28 (s, 1H), 8.24 (s, 1H), 7.46 (s, 1H), 6.97 (s, 2H), 4.94-4.76 (m, 3H), 4.63-4.55 (m, 1H), 4.34 (s, 1H), 4.09-4.01 (m, 1H), 3.31 (t, *J* = 6.64 Hz, 2H), 3.22 (s, 1H), 3.00-2.77 (m, 9H), 2.59 (t, *J* = 7.01 Hz, 4H), 1.88 (p, *J* = 6.89 Hz, 2H), 1.83-1.73 (m, 1H), 1.70-1.35 (m, 8H), 1.49 (s, 3H), 1.20-1.06 (m, 15H), 0.91-0.71 (m, 18H). ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm 208.14, 207.22, 172.12, 171.40, 170.57, 143.75, 140.05, 138.75, 123.71, 67.38, 58.99, 57.47, 57.44, 57.16, 52.12, 50.53, 50.41, 39.22, 38.07, 37.77, 36.92, 31.56, 27.65, 27.37, 25.29, 25.14, 24.88, 23.12, 22.85, 21.09, 17.35, 16.65, 15.20, 15.16, 11.51, 11.35. $[\alpha]_D^{23} = -0.57$ (*c* = 1, $CHCl_3$). LC-MS (System B): R_t (min): 10.66 (ESI-MS (m/z): 898.40 ($M + H^+$)). HRMS: calcd. for $C_{47}H_{75}N_7O_{10}$ 898.56482 $[M+ H]^+$; found 898.56539.

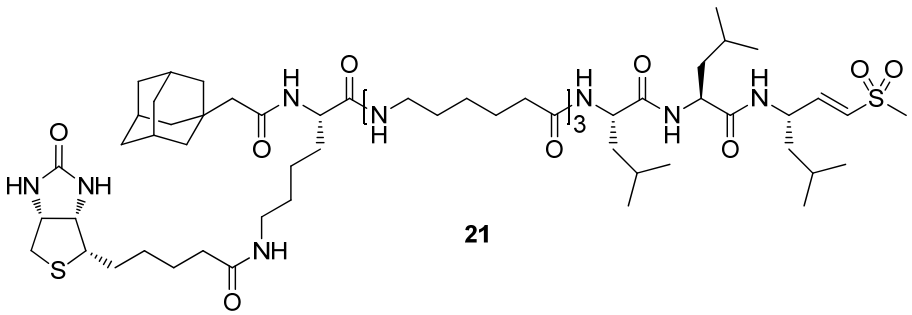
Biotin-Ahx-propargylamide (20)

Boc-Ahx-propargylamide[4] (275 mg, 1.03 mmol) was treated with a 1:1 v/v mixture of DCM/TFA (6 mL) for 1 h and subsequently coevaporated with toluene (3×). The resulting intermediate was dissolved in DMF (5 mL) and to this were added biotin-OSu (1.1 eq., 1.1 mmol, 375 mg) and DiPEA (1.5 eq., 1.50 mmol, 248 µL). The reaction was stirred for 14 h before being concentrated under reduced pressure. The title compound was obtained after crystallisation from MeOH/Et_2O as a colorless solid. The compound was sufficiently pure based on LC-MS analysis and subjected to the next step without further purification. LC-MS (System B): R_t (min): 4.27 (ESI-MS (m/z): 395.13 ($M + H^+$)).

Biotin-Ahx-triazole-Lev-phenol-epoxomicin (1)

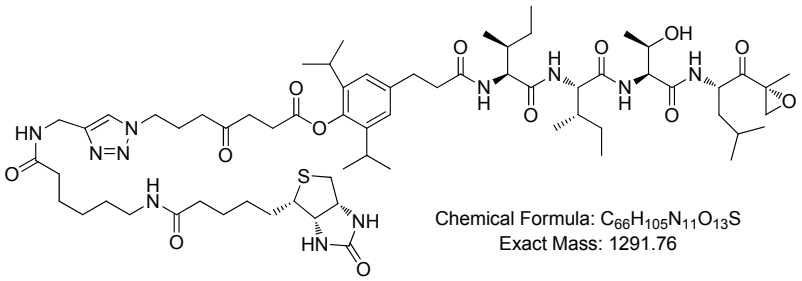
To a solution of compound **19** (45.0 mg, 50.1 µmol) and biotin-Ahx-propargylamide (**20**, 1.5 eq., 70.0 µmol, 30.0 mg) in DMF (1.5 mL) were added $CuSO_4$ (0.2 eq., 10.0 µmol, 10.0 µL; 1 M in H_2O) and sodium ascorbate (0.3 eq., 15.0 µmol, 15.0 µL; 1 M in H_2O) and the mixture was stirred for 14 h at RT after which LC-MS analysis indicated a complete conversion of the azide. The mixture was concentrated under reduced pressure and the title compound was obtained after RP-HPLC purification (10% → 90% ACN/0.1% aq. TFA) as a colorless solid (yield: 26.3 mg, 20.4 µmol, 41%). ^{1}H NMR (400 MHz, CD_3OD) δ ppm 7.76 (s, 1H), 6.92 (s, 2H), 4.45 (dd, *J* = 10.68, 3.02 Hz, 1H), 4.38 (dd, *J* = 7.43, 5.04 Hz, 1H), 4.34-4.26 (m, 4H), 4.22 (d, *J* = 5.04 Hz, 1H), 4.19 (dd, *J* = 7.87, 4.49 Hz, 1H), 4.15 (d, *J* = 7.99 Hz, 1H), 4.10 (d, *J* = 8.14 Hz, 1H), 3.93 (dd, *J* = 6.19, 5.28 Hz, 1H), 3.15 (d, *J* = 5.07 Hz, 1H), 3.12-3.08 (m, 1H), 3.05 (t, *J* = 7.07 Hz, 2H), 2.87-2.70 (m, 10H), 2.59 (d, *J* = 12.70 Hz, 1H), 2.51-2.39 (m, 4H), 2.15-2.01 (m, 6H), 1.81-1.70 (m, 1H), 1.69-1.37 (m, 12H), 1.36 (s, 3H), 1.35-1.17 (m, 6H), 1.10-1.01 (m, 16H), 1.00-0.88 (m, 1H), 0.86-0.77 (m, 12H), 0.76-0.69 (m, 6H). ^{13}C NMR (100 MHz, CD_3OD) δ ppm 209.52, 209.43, 176.00, 175.27, 174.10, 173.71, 173.61, 172.25, 145.32, 141.72, 140.24, 124.93, 68.57, 63.45, 61.69, 60.14, 59.84, 59.41, 59.32, 57.06, 53.10, 51.85, 50.58, 41.09, 40.37, 40.23, 39.53, 38.73, 38.04, 37.88, 37.79, 36.85, 35.64, 32.80, 30.16, 29.83, 29.54, 28.68, 28.61, 27.58, 26.98, 26.54, 26.26, 26.05, 25.46, 23.82, 21.53, 20.06, 17.06, 16.01, 15.97, 11.49, 11.37. $[\alpha]_D^{23} = -1.28$ (*c* = 1, MeOH). LC-MS (System B): R_t (min): 8.42 (ESI-MS (m/z): 1292.53 (M + H^+)). HRMS: calcd. for $C_{66}H_{105}N_{11}O_{13}S$ 1292.76868 $[M+H]^+$; found 1292.76980.

Structure of Ada-$(Ahx)_3$-Leu_2-LeuVS (AdaKBio, **21**)[5]

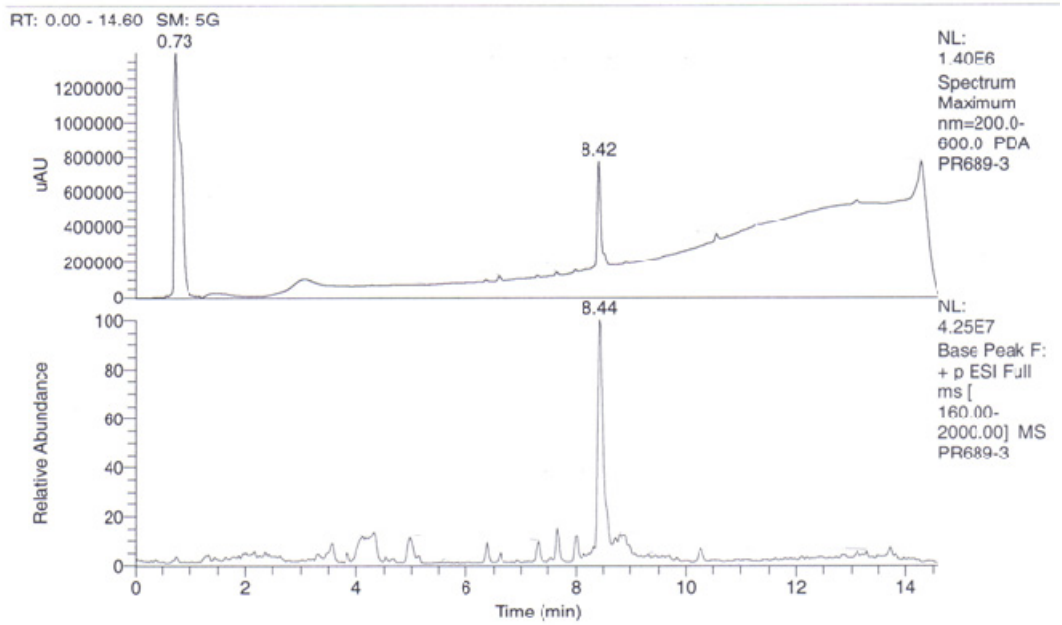


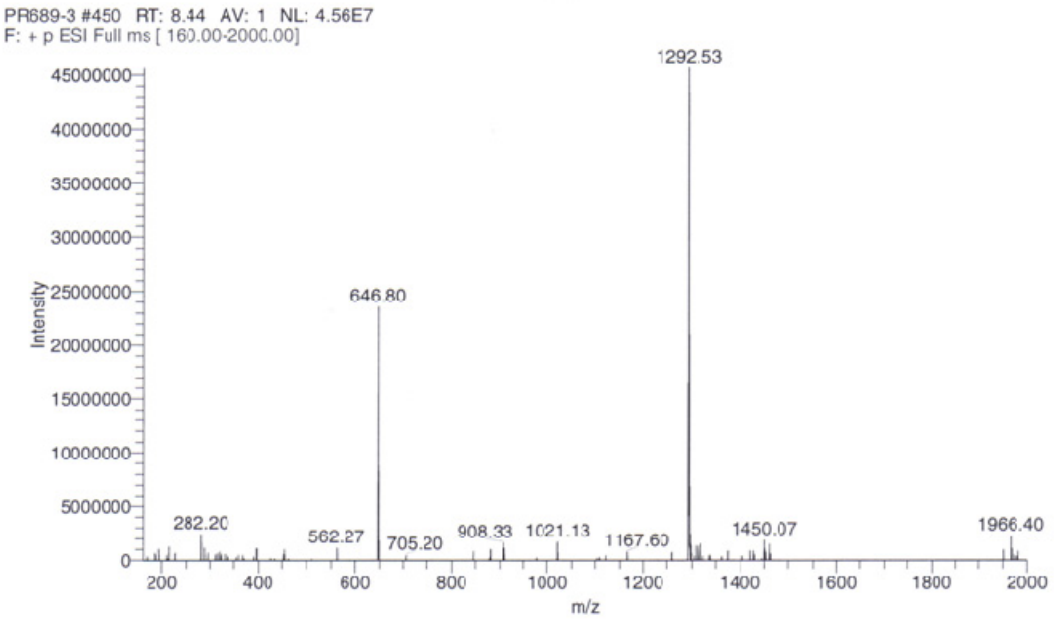
21

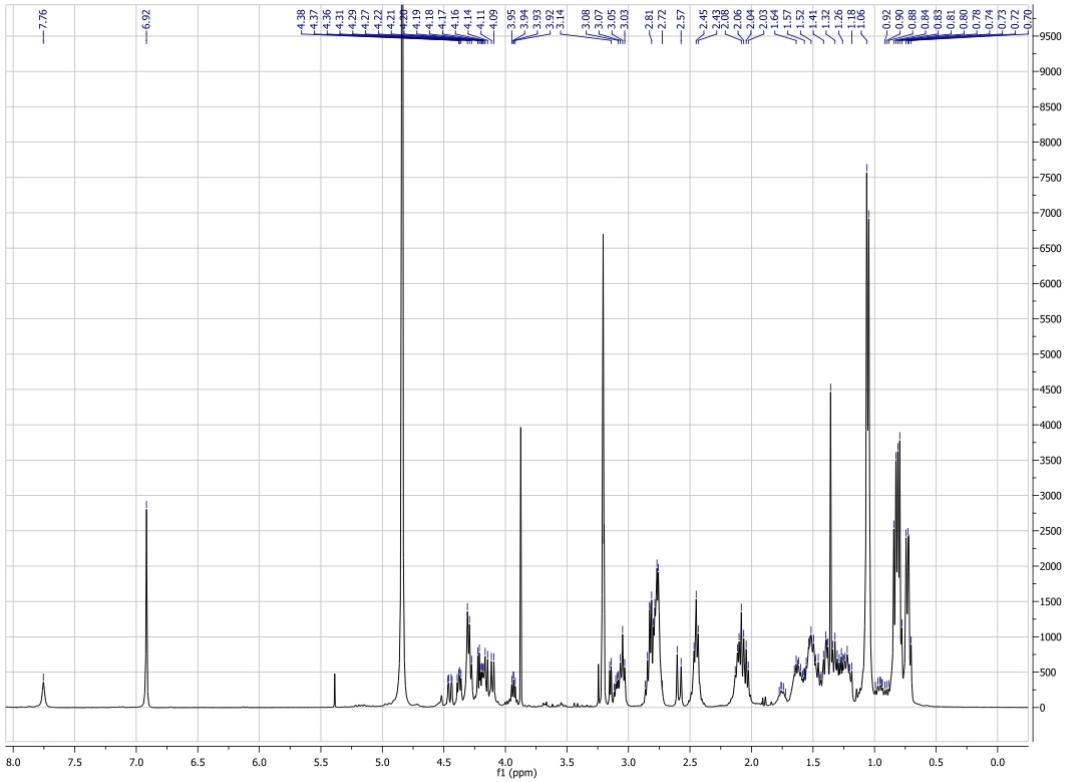
LC-MS and NMR spectra of compound 1

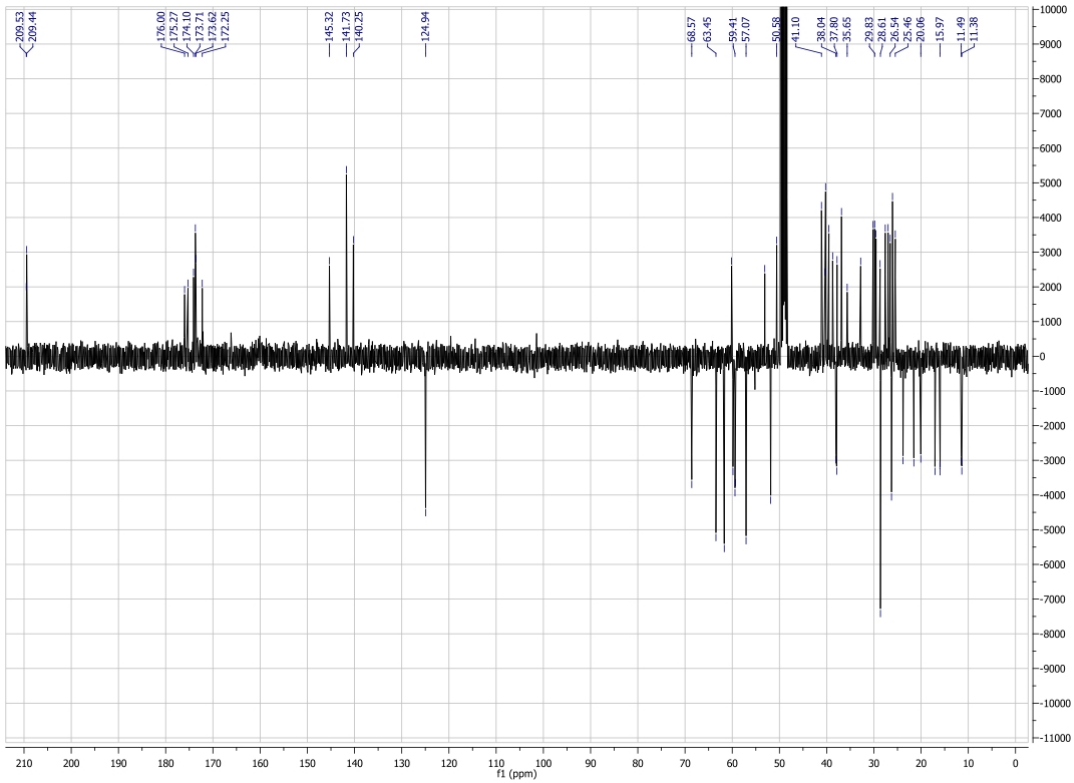


Chemical Formula: $C_{66}H_{105}N_{11}O_{13}S$
Exact Mass: 1291.76



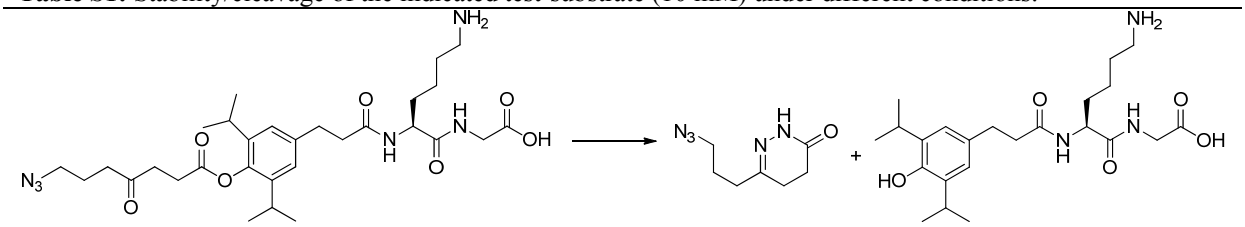



7.76
6.92
4.38
4.37
4.36
4.31
4.29
4.27
4.22
4.21
4.20
4.19
4.18
4.17
4.16
4.14
4.11
4.09
3.95
3.94
3.93
3.10
3.14
3.08
3.07
3.05
3.03
2.81
2.72
2.57
2.45
2.43
2.08
2.06
2.04
2.03
1.64
1.57
1.53
1.41
1.32
1.26
1.18
1.06
0.92
0.90
0.88
0.84
0.83
0.81
0.80
0.78
0.74
0.73
0.72
0.70
f1 (ppm)


209.53
209.44
176.00
175.27
174.10
173.71
173.63
172.25
145.32
141.73
140.25
124.94
68.57
63.45
59.41
57.07
50.58
41.10
38.04
37.80
35.65
29.83
28.61
26.54
25.46
20.06
15.97
11.49
11.38
f1 (ppm)

Stability check and optimization of the cleavage

Table S1: Stability/cleavage of the indicated test-substrate (10 mM) under different conditions.[a]



Entry	Buffer (pH)	H_2NNH_2	Temp. (ºC)	Time (h)	Additive	Cleavage
1	Tris (7.5)	–	23	15	–	–
2	Tris (7.5)	–	37	15	–	–
3	Tris (7.5)	+	23	15	–	+
4	Tris (7.5)	+	37	1	–	+
5	Tris (7.5)	+	37	15	–	+
6	Tris (7.5)	NH_2OH	37	15	–	–
7	Tris (7.5)	–	23	15	0.4% SDS	–
8	Tris (7.5)	–	100	5 min.	4×SB	–
9	HEPES (5.8)	–	23	15	–	–
10	PBS (7.4)	–	23	15	–	–
11	MI (3.0)	–	23	15	–	–
12	MI (4.0)	–	23	15	–	–
13	MI (5.0)	–	23	15	–	–
14	MI (6.0)	–	23	15	–	–

[a] Concentrations used: [Substrate] = 10 mM; [H_2NNH_2] = [NH_2OH] = 100 mM; [Tris] = 100 mM; [HEPES] = 50 mM. PBS (phosphate buffered saline): 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 0.14 M NaCl, 2.7 mM KCl. MI (McIlvaine's buffers): combinations of 0.1 M citric acid and 0.2 M Na_2HPO_4. SB (sample buffer): 20 mM Tris, 1% SDS, 10% glycerol, 0.05% BPB, β-mercaptoethanol.

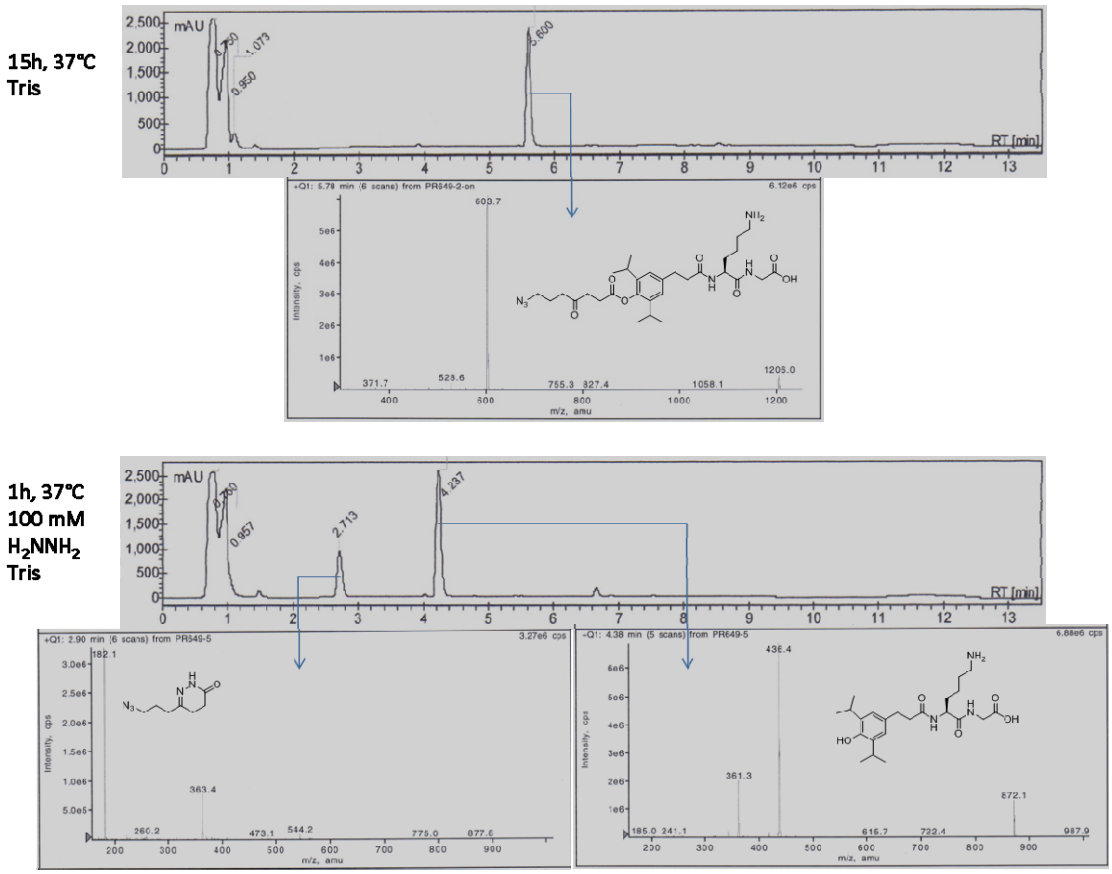


Figure S1: LC-MS spectra of samples from entry 2 and 5 (Table S1), gradient: 10% → 90% ACN/0.1% aq. TFA.

Optimization of the cleavage: hydrazine concentration & temperature

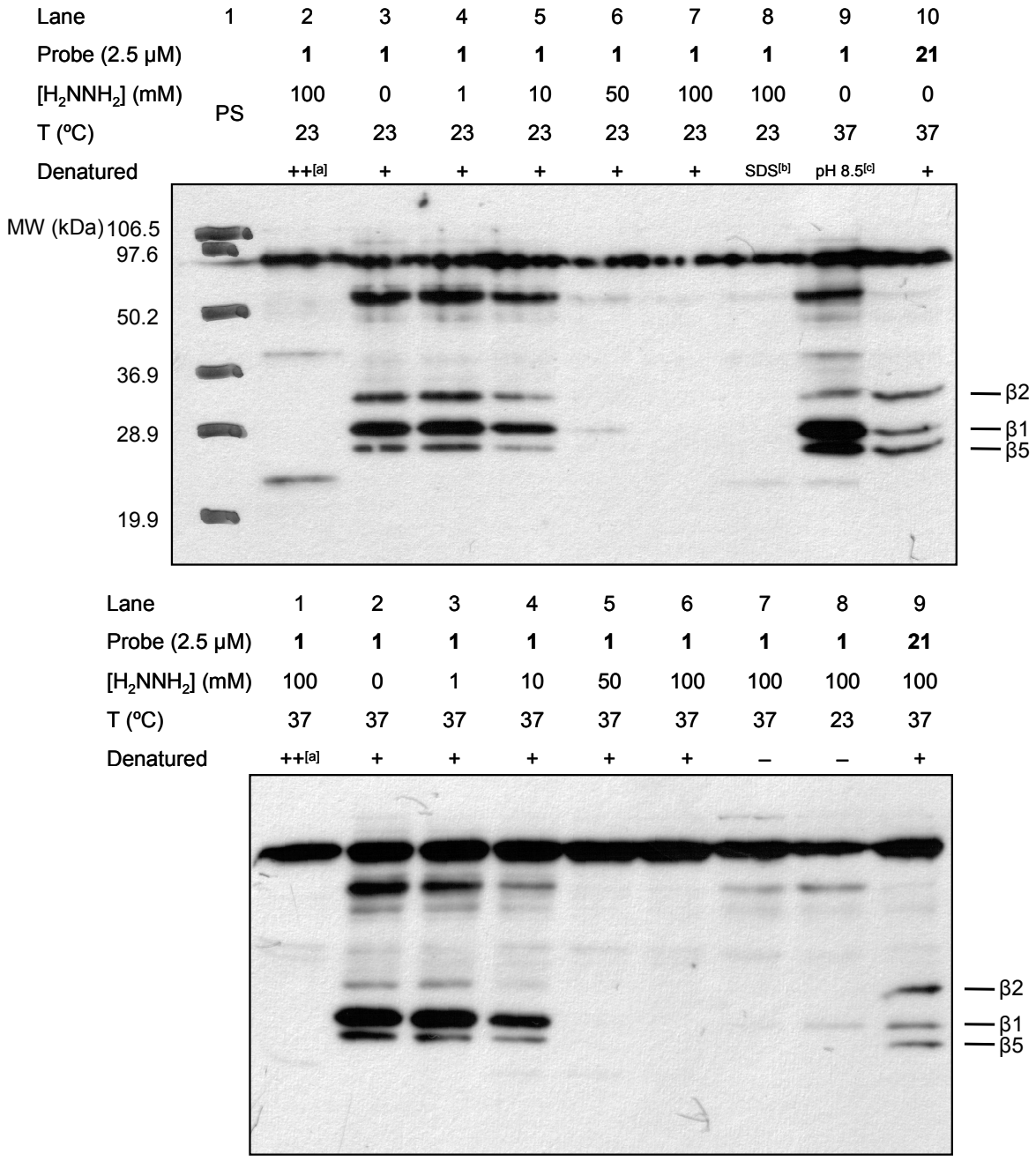


Figure S2: Search for the optimal cleavage conditions in a biological environment. HEK-293T cell lysate was incubated with probe **1** or **21** (2.5 µM final concentration) for 1 h at 37 ºC followed by denaturation (except for lanes 7 and 8 of the lower gel). Samples were treated with the indicated hydrazine concentration for 15 h, resolved by SDS-PAGE and all biotinylated proteins were visualized by anti-biotin Western blotting. Cleavage of the linker is shown by disappearance of the bands. PS: prestained marker low range (Bio-Rad). [a] Samples were pre-boiled with 1% SDS prior to incubation as a negative control. [b] 20 mM SDS without denaturing. [c] pH 8.5 is the pH value for a combination of 100 mM Tris and 100 mM hydrazine and was reached by addition of 1 µL 1 M Tris to the sample.

Optimization of the cleavage: time dependence

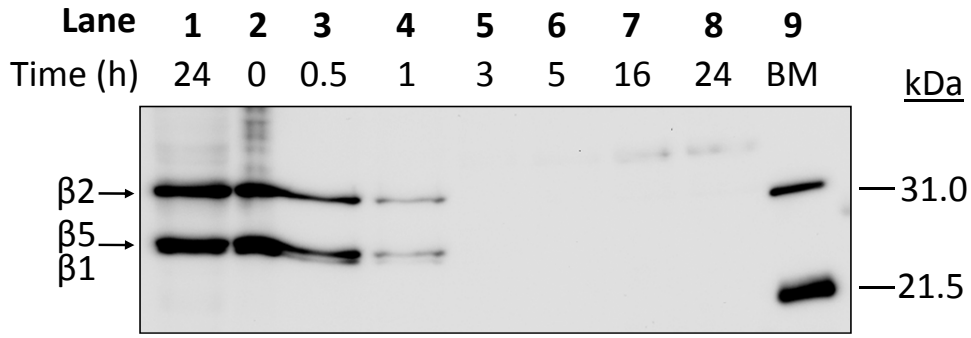


Figure S3: Time dependence of the hydrazine mediated cleavage in a biological environment. HEK-293T cell lysate was incubated with 2.5 µM (final concentration) probe **1** (Lanes 2-8) or **21** (lane 1) for 1 h at 37 ºC followed by denaturation. Samples were treated with hydrazine (100 mM) for 0.5, 1, 3, 5, 16 or 24 hours, resolved by SDS-PAGE and all biotinylated proteins were visualized by anti-biotin Western blotting. Cleavage of the linker is shown by disappearance of the bands. BM: biotinylated marker low range (Bio-Rad). Lane 2: no hydrazine was added.

Pull-down experiments

General procedure

HEK-293 cell lysate (containing some 1.3 mg of protein) was incubated with 20 µM ABPs **1** or **21** for 1 h at 37 °C, denatured by boiling for 5 min with 1% SDS and precipitated with chloroform/methanol (C/M).[6] The protein pellet was rehydrated in 180 µL 8 M urea/100 mM NH_4HCO_3, reduced with 10 µL 90 mM DTT for 30 min at 37 °C, alkylated with 15 µL 200 mM iodoacetamide at RT in the dark, cleared by centrifugation at 13,000 g and desalted by C/M. The pellet was dispersed in 25 µL pull down (PD) buffer (50 mM Tris·HCl pH 7.5, 150 mM NaCl) with 2% SDS in a heated (37 °C) sonic bath. Stepwise (3 × 25 µL, 4 × 100 µL, 1 × 500 µL) addition of PD buffer afforded a clear solution that was incubated with 50 µL MyOne T1 Streptavidin grafted magnetic beads (Invitrogen) at RT with vigorous shaking for 1 h. The beads were stringently washed with 2 × 300µL PD buffer with 0.1% SDS, 2 × 300 µL PD buffer, 2 × 300 µL wash buffer I (4 M urea/50 mM NH_4HCO_3), 2 × 300 µL wash buffer II (50 mM Tris·HCl pH 7.5, 10 mM NaCl) and 2 × 300 µL water. All 5 samples from ABP **1** were mixed and divided over 8 equal portions. The same was done for the 2 samples from ABP **21**, which were divided over 3 equal portions. To the samples was added 65 µL of the appropriate cleavage cocktail (see Figure 2 in the article) and the samples were shaken for 15 h at RT or at 37 ºC. Next, the supernatant was removed, diluted with 20 µL 4× sample buffer and boiled at 100 ºC for 5 min. One sample from ABP **21** was treated directly with 85 µL 1× sample buffer containing 10 µM biotin, boiled at 100 ºC for 5 min and stored o/n at 4 ºC. The samples were resolved by 12.5% SDS-PAGE (25 µL of each sample was loaded) and all proteins were visualized by silverstain. The appropriate bands were cut from the gel and an in-gel digestion was performed according to the procedure described in literature.[7]

After removal of the supernatant all beads were stringently washed as described above, eluted with 85 µL 1× sample buffer containing 10 µM biotin at 100 ºC for 5 min., resolved on SDS-PAGE and visualized by silverstain to determine the cleavage efficiency (see Figure S4).

LC-MS/MS analysis

Trytic peptides were analyzed on a Surveyor nanoLC system (Thermo) hyphenated to a LTQ-Orbitrap mass spectrometer (Thermo). Gold and carbon coated emitters (OD/ID = 360/25 µm tip ID = 5 µm), trap column (OD/ID = 360/100 µm packed with 25 mm robust Poros®10R2/15 mm BioSphere C18 5 µm 120 Å) and analytical columns (OD/ID = 360/75 µm packed with 20 cm BioSphere C18 5 µm 120 Å) were from Nanoseparations (Nieuwkoop, The Netherlands). The mobile phases (A: 0.1% FA/H_2O, B: 0.1% FA/ACN) were made with ULC/MS grade solvents (Biosolve). The emitter tip was coupled end-to-end with the analytical column via a 15 mm long TFE teflon tubing sleeve (OD/ID 0.3 × 1.58 mm, Supelco, USA) and installed in a stainless steel holder mounted in a nanosource base (Upchurch scientific, Idex, USA). General mass spectrometric conditions were: an electrospray voltage of 1.8 kV was applied to the emitter, no sheath and auxiliary gas flow, ion transfer tube temperature 150 ºC, capillary voltage 41 V, tube lens voltage 150 V. Internal mass calibration was performed with air-borne protonated polydimethylcyclosiloxane (*m/z* = 445.12002) and the plasticizer protonated dioctyl phthalate ions (*m/z* = 391.28429) as lock mass.[8] For shotgun proteomics analysis, 10 µL of the samples was pressure loaded on the trap column with a 10 µL/min flow for 5 min. followed by peptide separation with a gradient of 35 min. 5 → 30% B, 15 min. 30 → 60% B, 5 min. A at a flow of 300

μL/min split to 250 nL/min by the LTQ divert valve. For each data dependent cycle, one full MS scan (300-2000 *m/z*) acquired at high mass resolution (60,000 at 400 *m/z*, AGC target 1×10^6, maximum injection time 1,000 ms) in the Orbitrap was followed by 3 MS/MS fragmentations in the LTQ linear ion trap (AGC target 5×10^3, max injection time 120 ms) from the three most abundant ions.[9] MS/MS settings were: collision gas pressure 1.3 mT, normalized collision energy 35%, ion selection threshold of 500 counts, activation q = 0.25 and activation time of 30 ms. Fragmented precursor ions that were measured twice within 10 s were dynamically excluded for 60 s and ions with $z < 2$ or unassigned were not analyzed. Data from MS/MS was validated manually. The results are shown in Table S2 and in the LC-MS/MS spectra for the active site fragments of the β2 and β5 subunits.

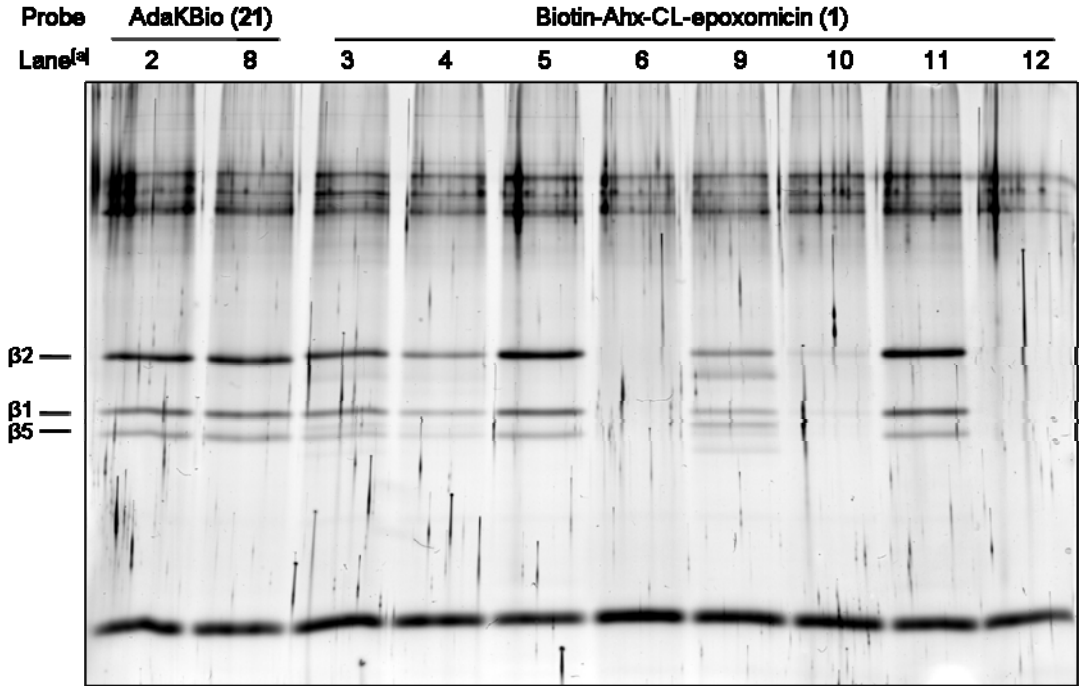


Figure S4: Determination of the cleavage efficiency. The beads from the samples in Figure 2 after hydrazine mediated cleavage were washed extensively and boiled for 5 min. with sample buffer (20 mM Tris, 1% SDS, 10% glycerol, 0.05% BPB, β-mercaptoethanol) containing 10 μM biotin. Samples were resolved by SDS-PAGE and all proteins were visualized by silverstain. [a] The numbers correspond to the same samples in the lanes in Figure 2 of the article.

Table S2: Identified proteasome β-subunit peptides after pull-down and tryptic digest.[a]

Accession #		Mass (Da)	Sequence coverage	*z*	ppm	Peptide score	Peptide sequence
IPI00000811	(β1)	21890	39.0%	2	0.90	25.8	FAVATLPPA #
				2	0.37	49.0	QVLLGDQIPK
				2	0.90	40.7	QVLLGDQIPK
				2	6.21	43.9	QVLLGDQIPK
				2	0.81	85.0	LAAIAESGVER
				2	0.10	98.6	EECLQFTANALALAMER
				2	0.50	96.3	EECLQFTANALALAMER
				3	0.00	71.1	YREDLMAGIIIAGWDPQEGGQVYSVPMGGMMVR *
IPI00003217	(β2)	25279	42.7%	2	0.69	78.1	DGIVLGADTR
				3	0.51	51.0	FRPDMEEEEAK
				2	0.14	51.9	LDFLRPYTVPNK
				2	1.01	144.9	LPYVTMGSGSLAAMAVFEDK
				2	2.00	104.9	LPYVTMGSGSLAAMAVFEDK
				3	2.47	62.4	LPYVTMGSGSLAAMAVFEDK
				2	0.00	118.4	LPYVTMGSGSLAAMAVFEDK
				2	1.12	99.6	ITPLEIEVLEETVQTMDTS #
				2	2.31	102.1	ITPLEIEVLEETVQTMDTS #
				2	0.42	119.1	NLVSEAIAAGIFNDLGSGSNIDLCVISK
IPI00479306	(β5)	22444	54.4%	2	1.45	75.2	LLANMVYQYK
				2	0.72	73.3	LLANMVYQYK
				2	0.08	94.0	ATAGAYIASQTVK
				2	1.33	79.7	GPGLYYVDSEGNR
				2	0.20	97.7	DAYSGGAVNLYHVR
				2	0.48	96.1	GYSYDLEVEQAYDLAR
				2	0.71	71.8	ISGATFSVGSGSVYAYGVMDR
				2	1.73	115.6	ISGATFSVGSGSVYAYGVMDR
				3	1.39	61.0	VIEINPYLLGTMAGGAADCSFWER

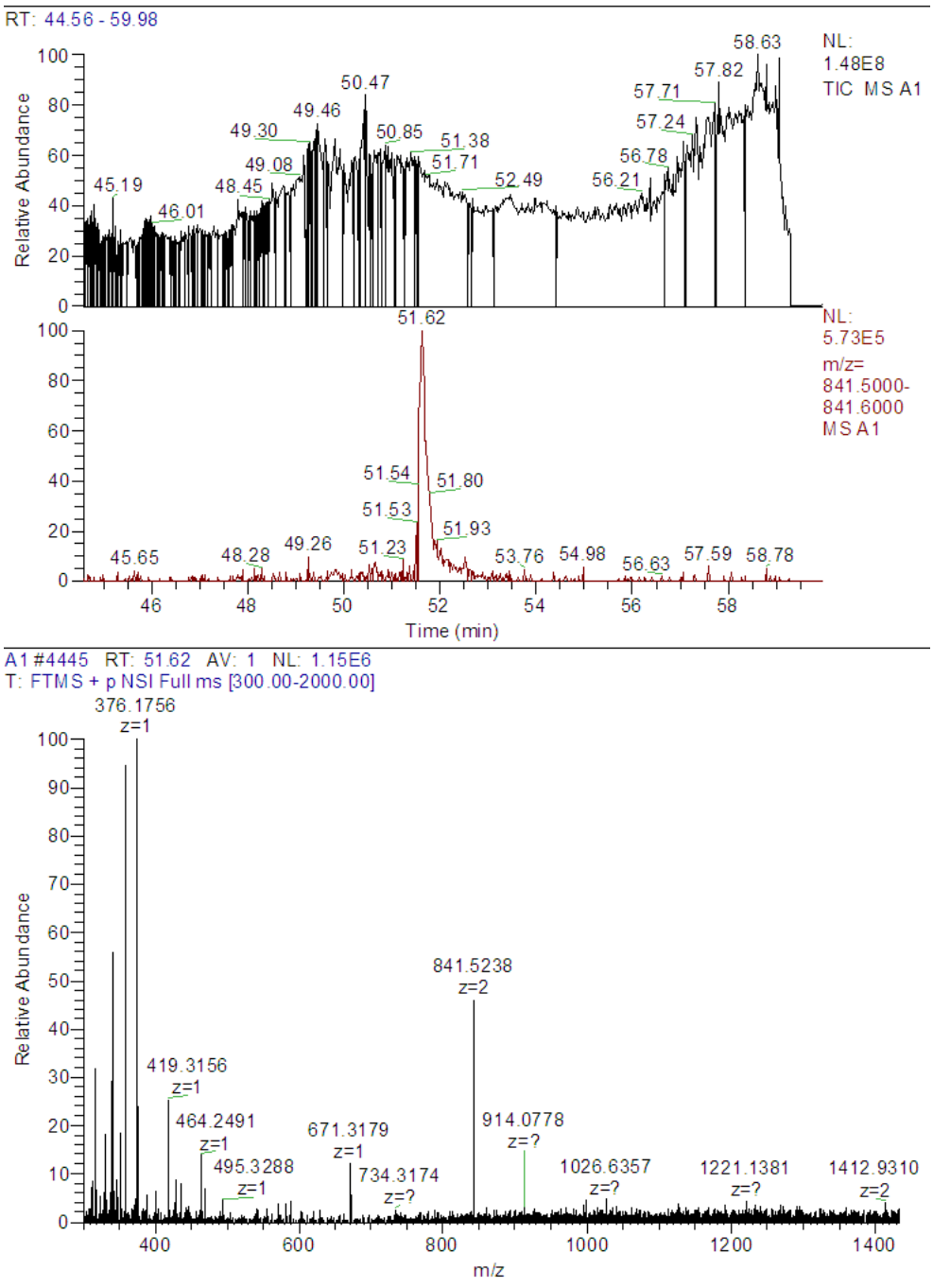
[a] Protein name, mass of the active β subunit, % coverage of the protein by amino acids identified by LC-MS, charge of the peptide (*z*), measurement error (ppm), Mascot peptide scores, miss cleavage (*), and C-terminal peptides (#). Mascot identifications were manually validated.

Table S3: Calculated exact (*m/z*) masses of the active-site peptides bound to compound **1** after cleavage.[a]

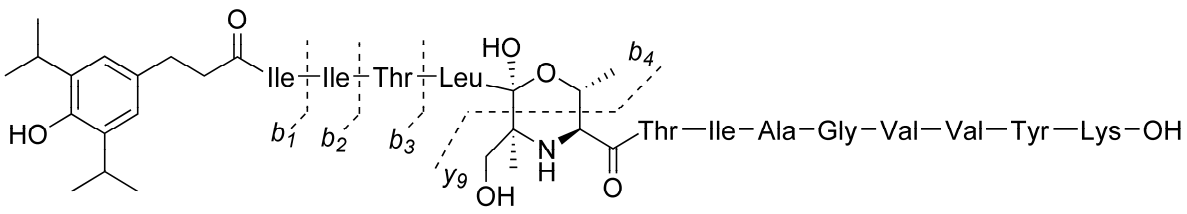
		Exact mass		*z*=1		*z*=2		*z*=3	
		mono-iso	High-peak	mono-iso	High-peak	mono-iso	High-peak	mono-iso	High-peak
β1	$C_{123}H_{203}N_{27}O_{36}S$	2666.4605	2667.4638	2667.4678	2668.4711	1334.2375	1334.7392	889.8274	667.8732
β2	$C_{84}H_{140}N_{14}O_{21}$	1681.0317		1682.0390		841.5232	842.0248	561.3512	
β5	$C_{76}H_{126}N_{12}O_{19}$	1510.9262		1511.9335		756.4704		504.6493	

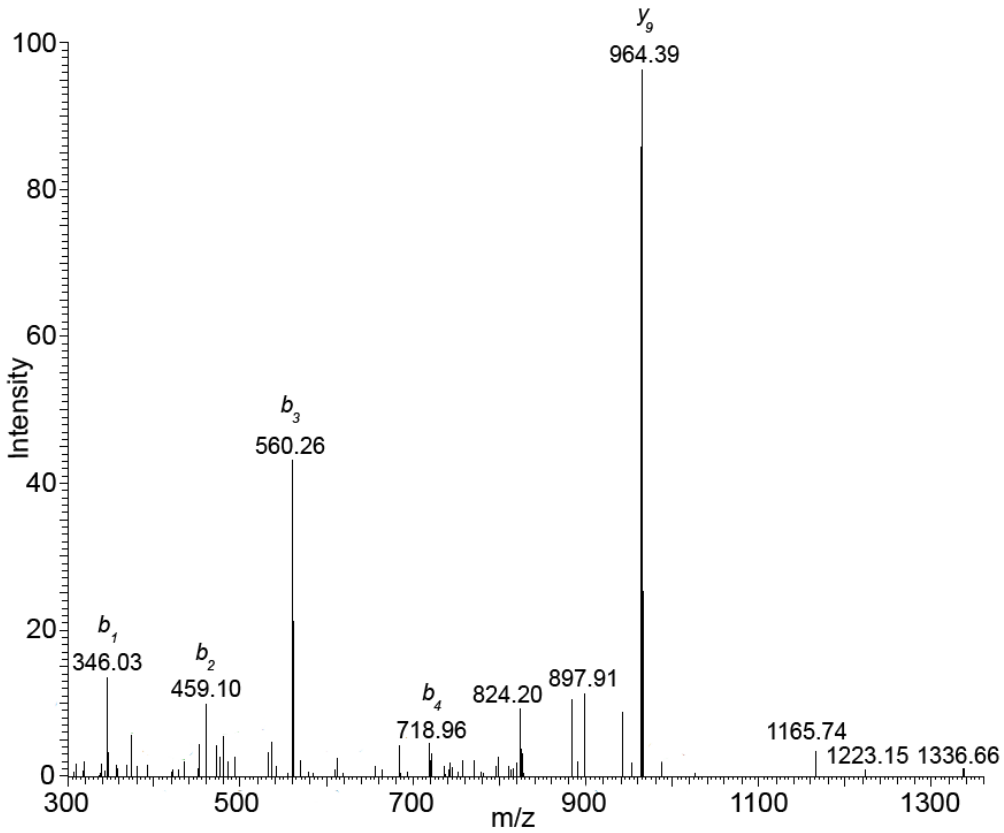
[a] The mono-isotopic (mono-iso) and the mass of the most abundant (High-peak) are shown at charge (*z*) of 1, 2 and 3. The missing high peaks are absent because the mono-isotopic peak is also the highest peak.

LC-MS of the enriched β2 subunit active site

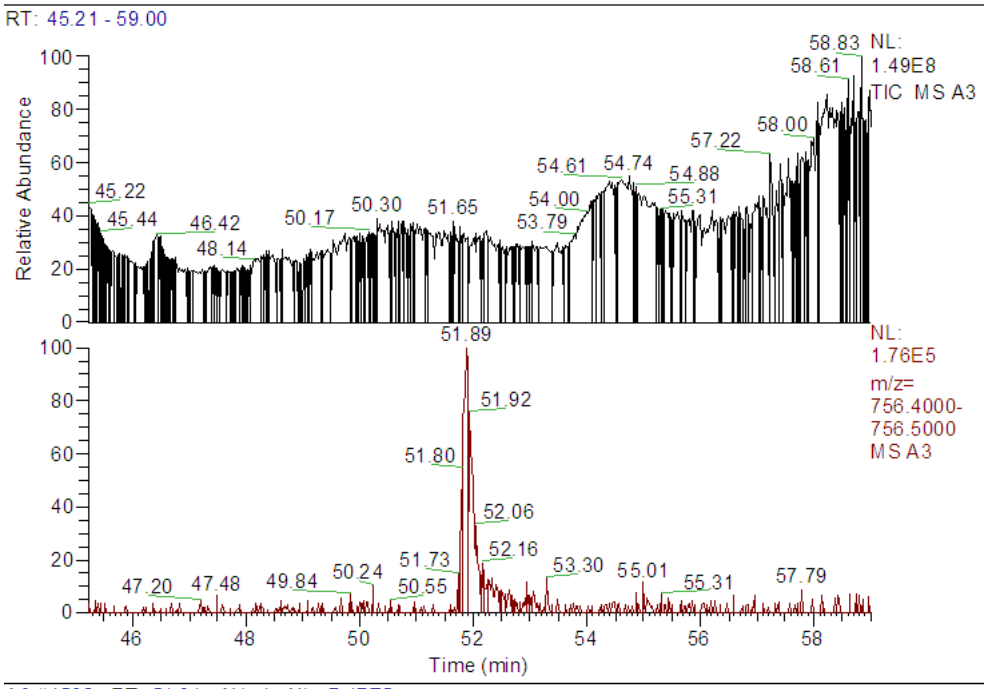


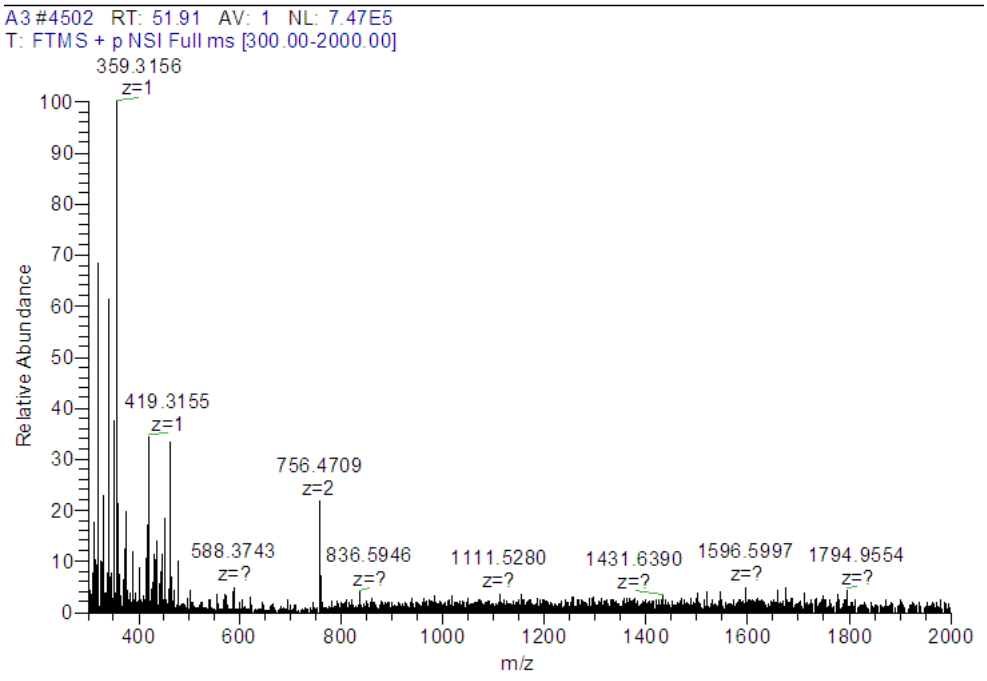
MS/MS of the β2 active site fragment



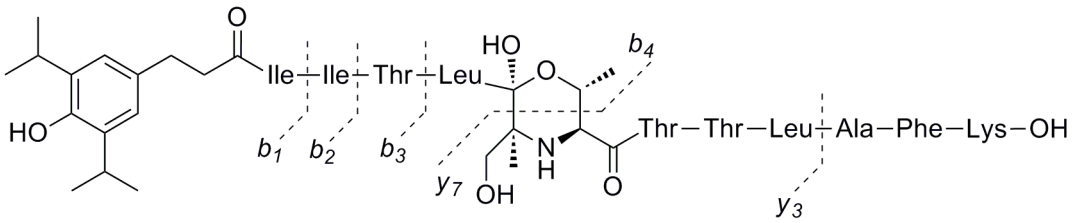


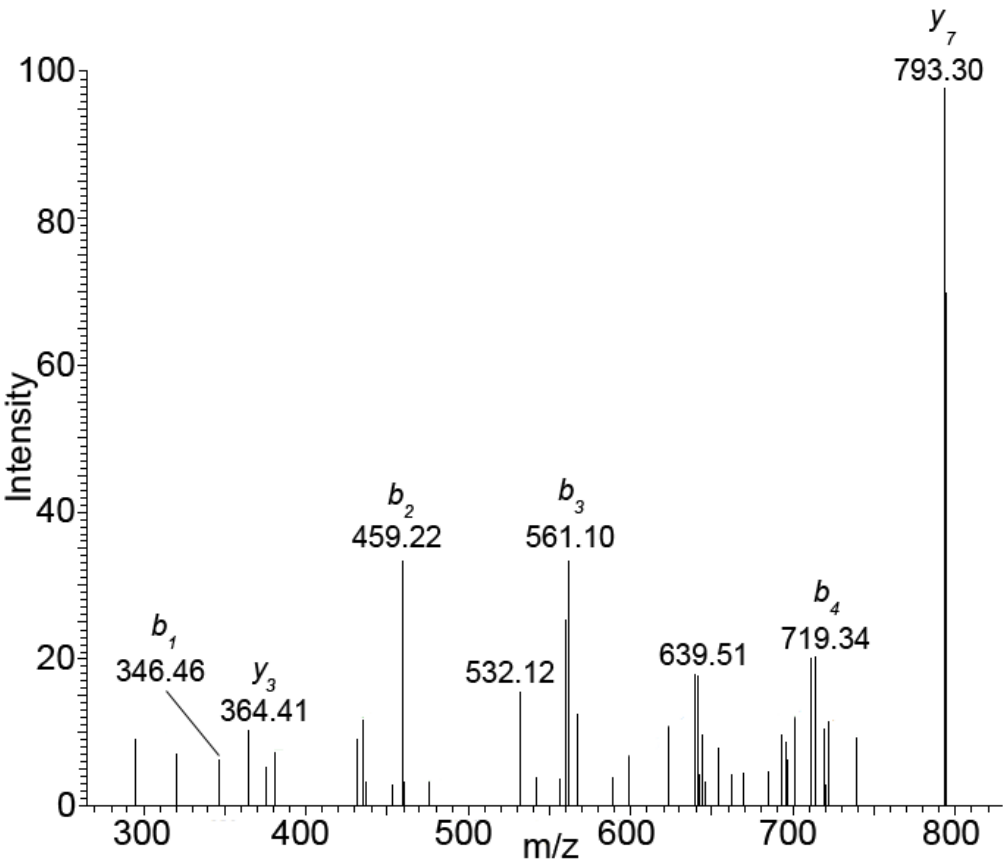
LC-MS of the enriched β5 subunit active site



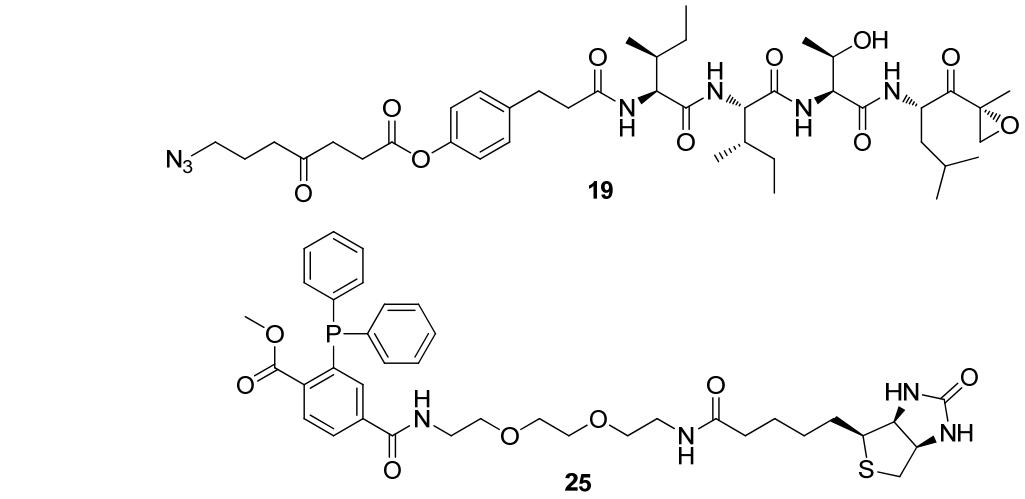


MS/MS of the β5 active site fragment





Labeling of proteasome active subunits with compound 19 followed by Staudinger-Bertozzi ligation with biotin-phosphane 25[10]



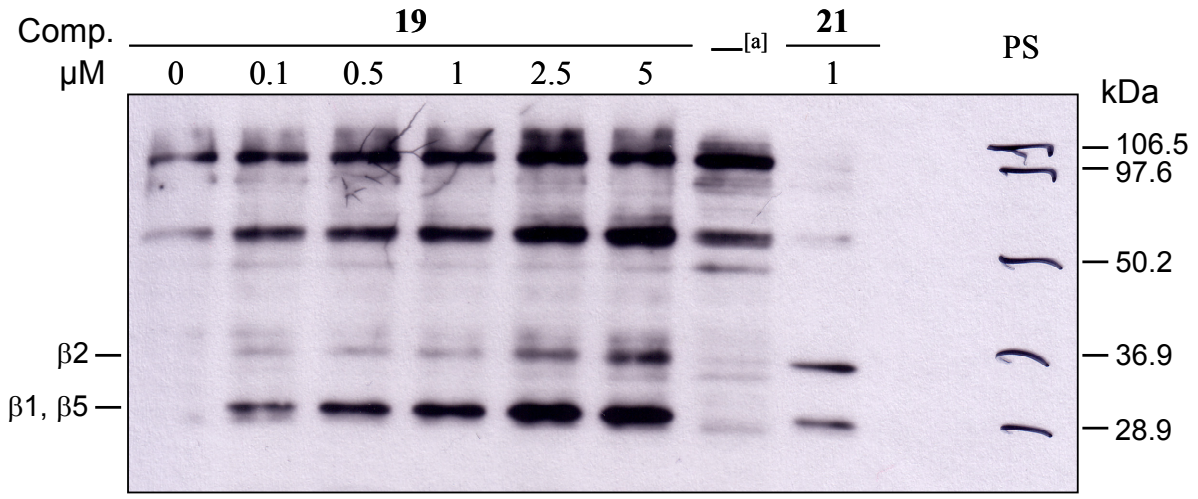
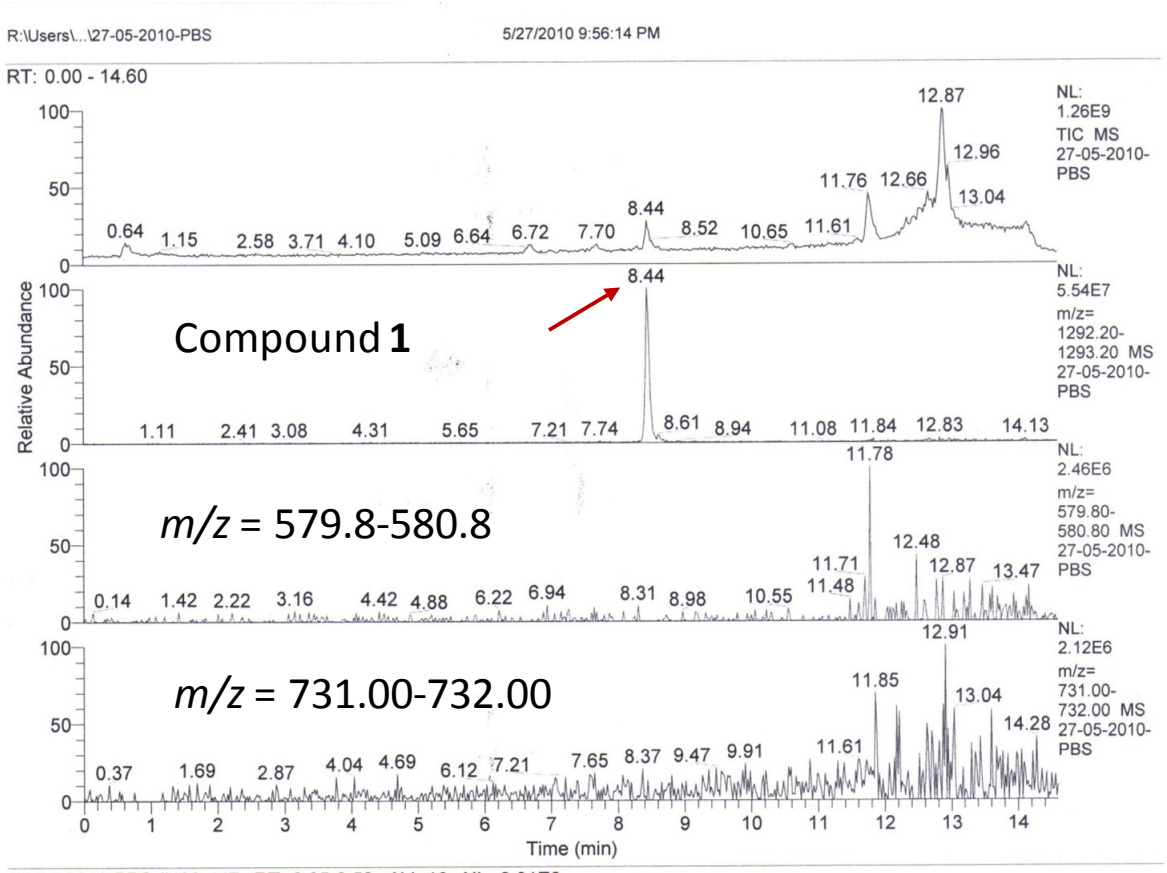


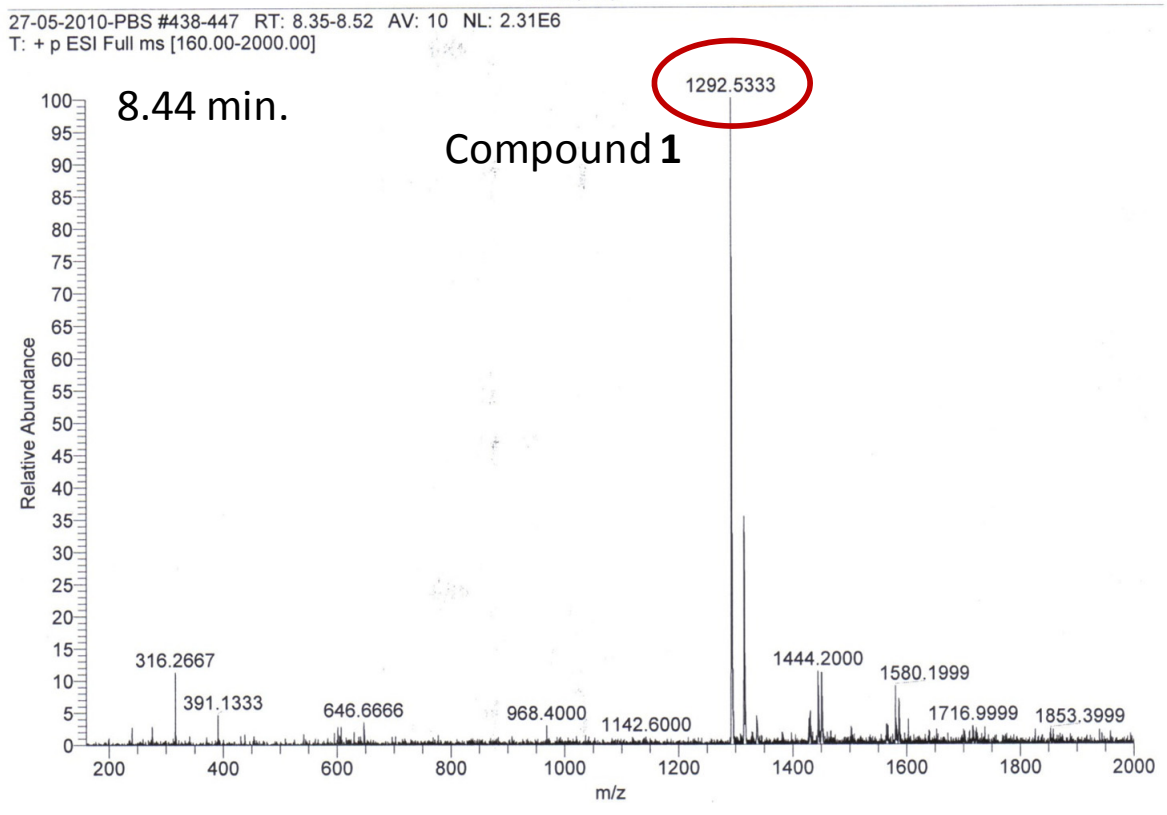
Figure S5: HEK-293 cell lysate (some 13.5 µg) was incubated with indicated final concentrations of compound **19** or AdaKBio (**21**) for 1 h at 37 ºC followed by Staudinger-Bertozzi ligation (400 µM final concentration biotin-phosphane **25**,[10] 2 h at 37 ºC) and SDS-PAGE. Read-out by streptavidin Western blotting. PS = prestained marker low range (Bio-Rad). [a] Negative control: sample was denatured (5 min. at 100 ºC with 1% SDS) prior to incubation with **19** (1 µM) and Staudinger-Bertozzi ligation.

Stability of the cleavable linker in blood serum and plasma

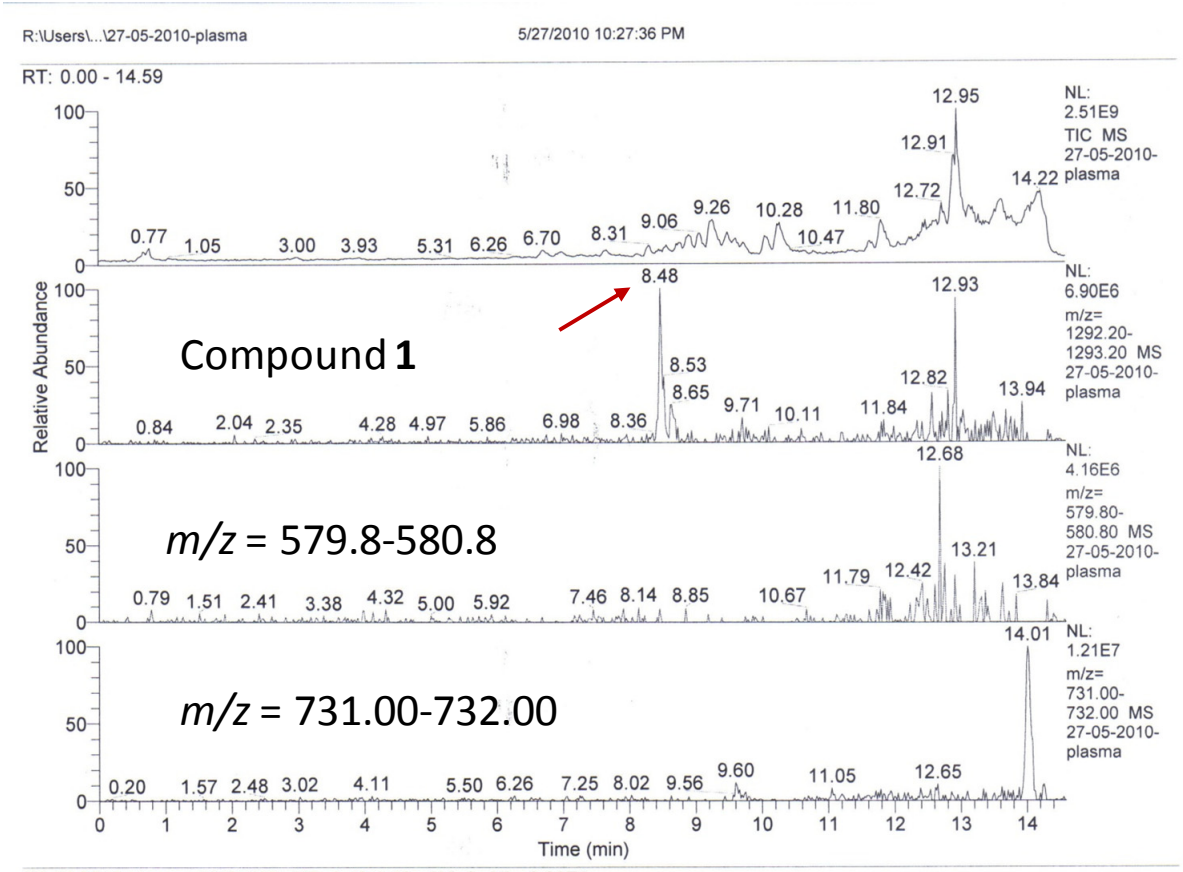
Plasma and serum were collected from blood voluntarily donated by a healthy person. For plasma collection: blood was extracted in an EDTA tube and centrifuged for 15 min. at 4,000 g. For serum collection no EDTA was used. All experiments were executed in duplo. A volume of 100 µL plasma or serum was incubated with compound **1** (10 µM final concentration) with or without hydrazine (100 mM final concentration) for 15 h at 37 °C. Next, 900 µL of cooled (–20 °C) acetone was added and the samples were kept at –20 °C for 2 h and an additional 1 h at –80 °C. This was followed by centrifuging of the samples for 10 min. at 14,000 g, after which all acetone was evaporated under reduced pressure. The resulting yellowish pellet was extracted with 100 µL ACN/H_2O/*t*BuOH (1:1:1) for 5 min. and the samples were analyzed by LC-MS (injection of 20 µL of the extract, system B, 10% → 90% ACN/0.1% aq. TFA). The LC-MS spectra are shown below. No products resulting from hydrolysis of the ester linkage could be detected.

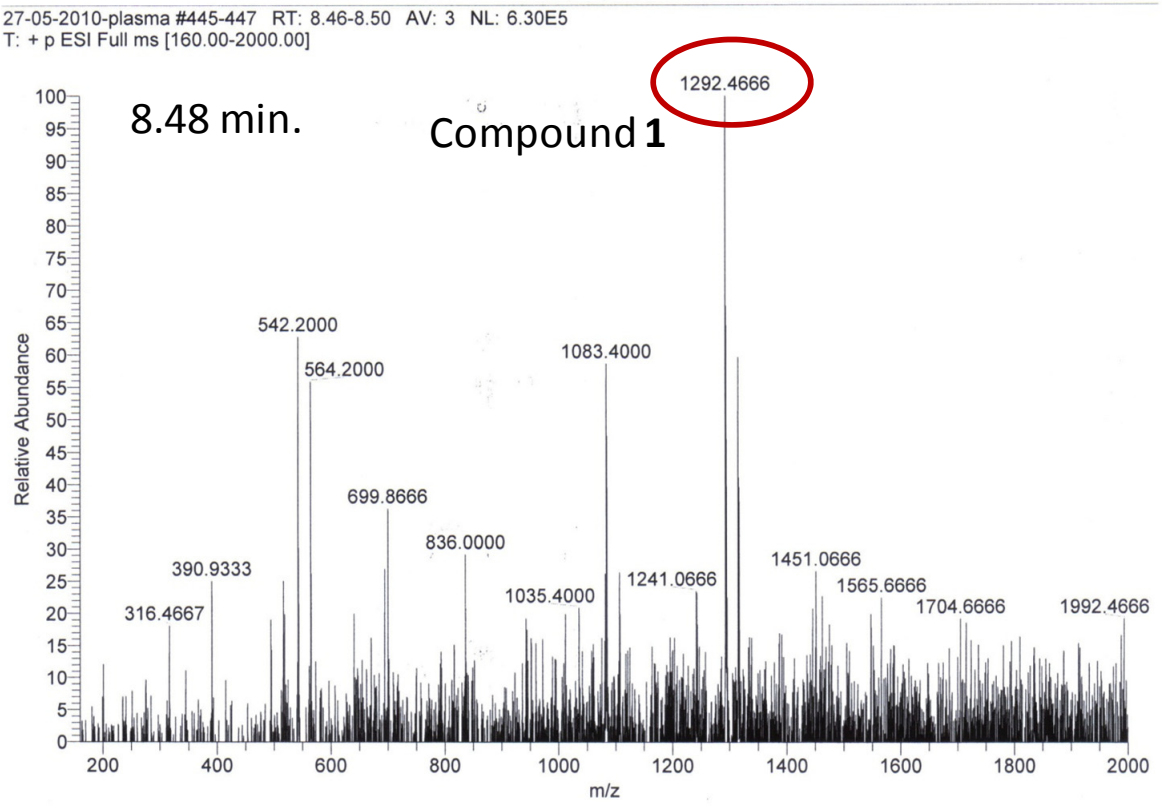
PBS + compound **1**



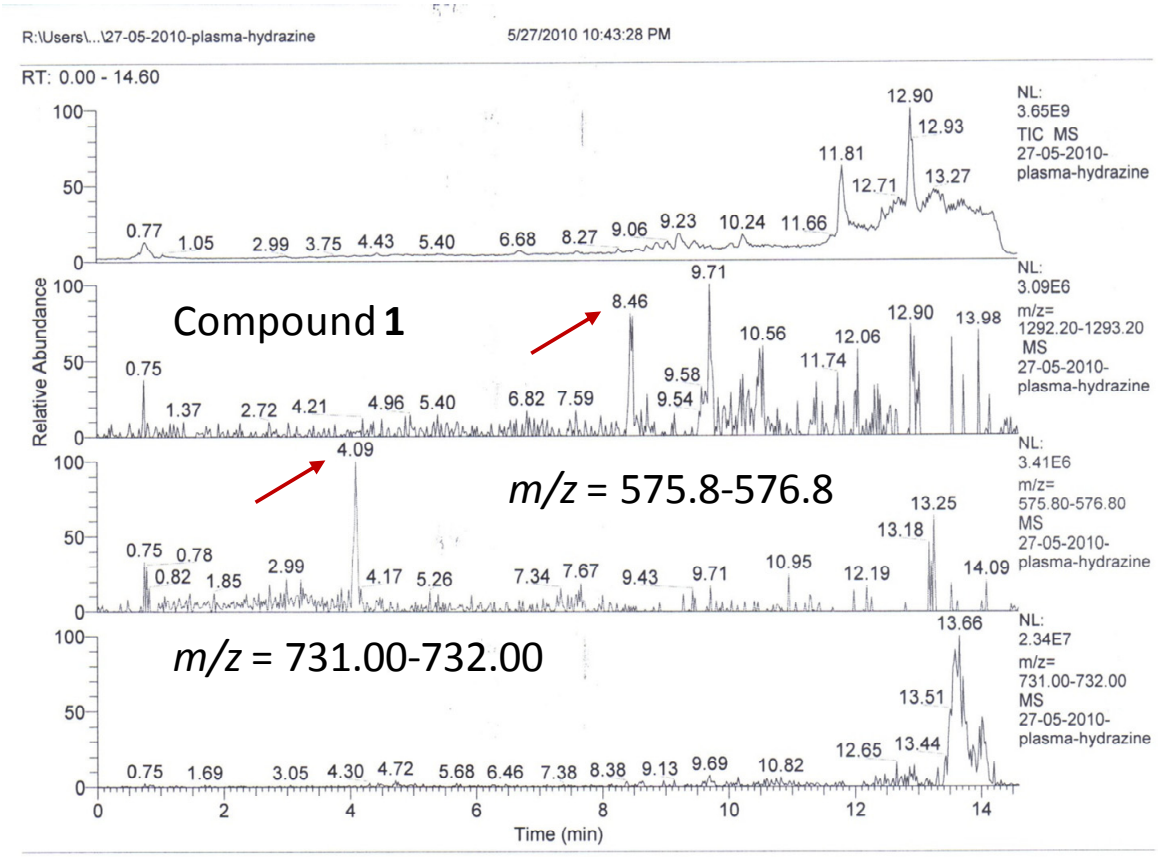


plasma + compound **1**

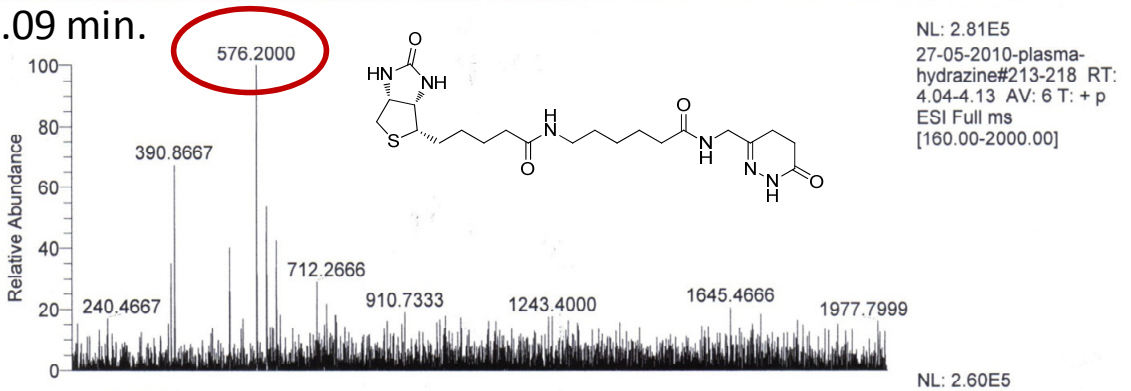


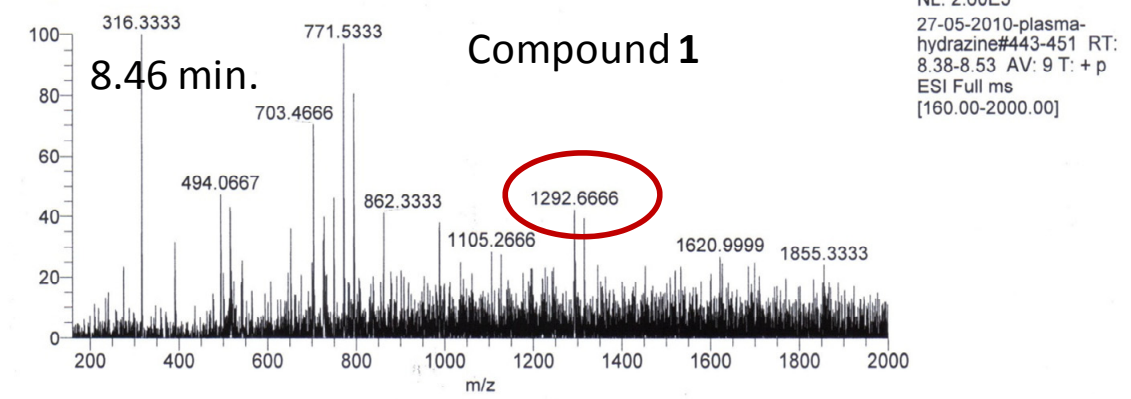


plasma + compound **1** + H_2NNH_2

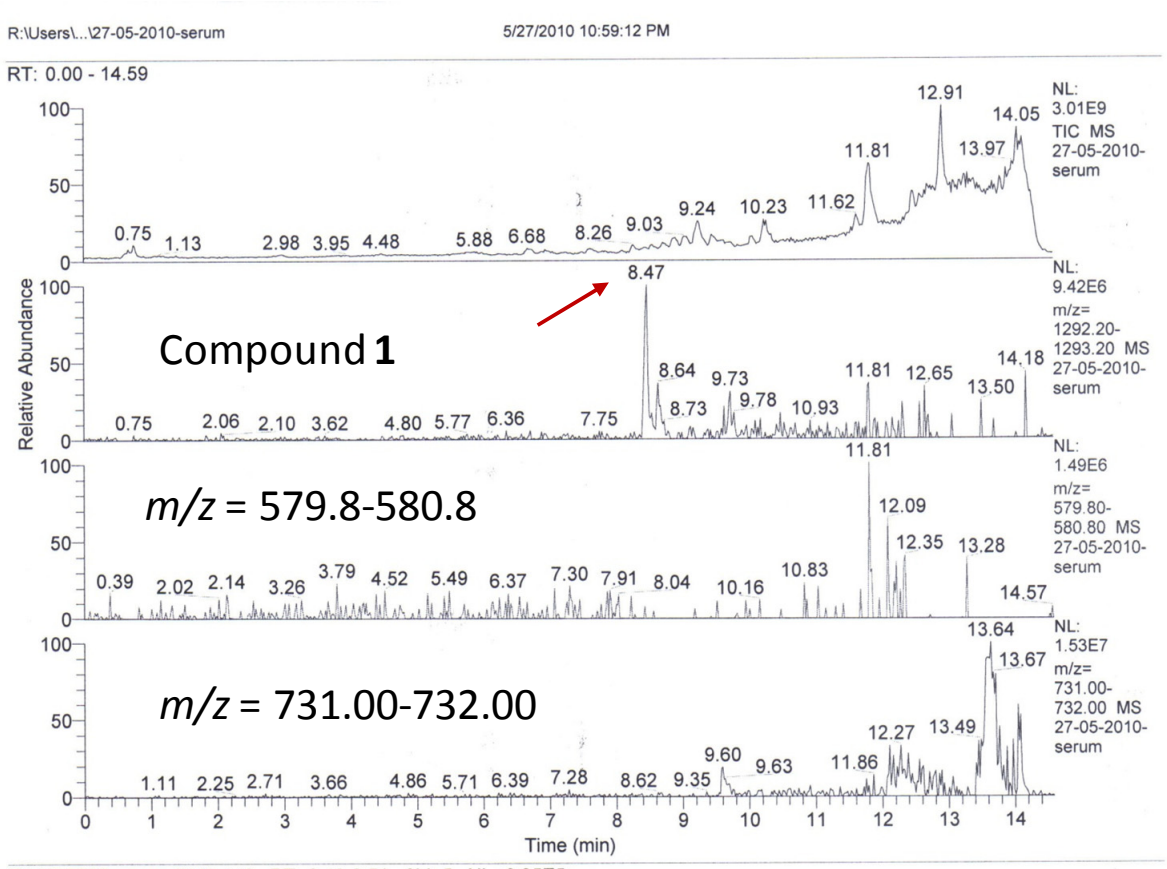


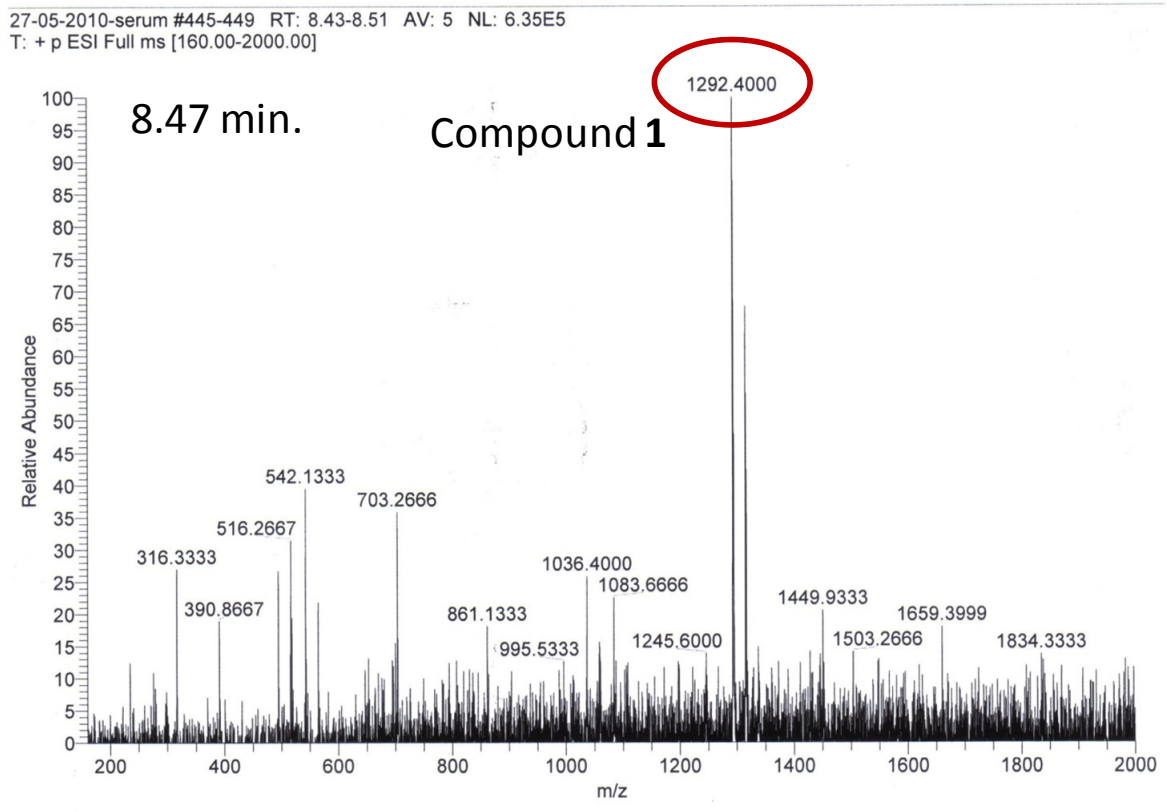
4.09 min.



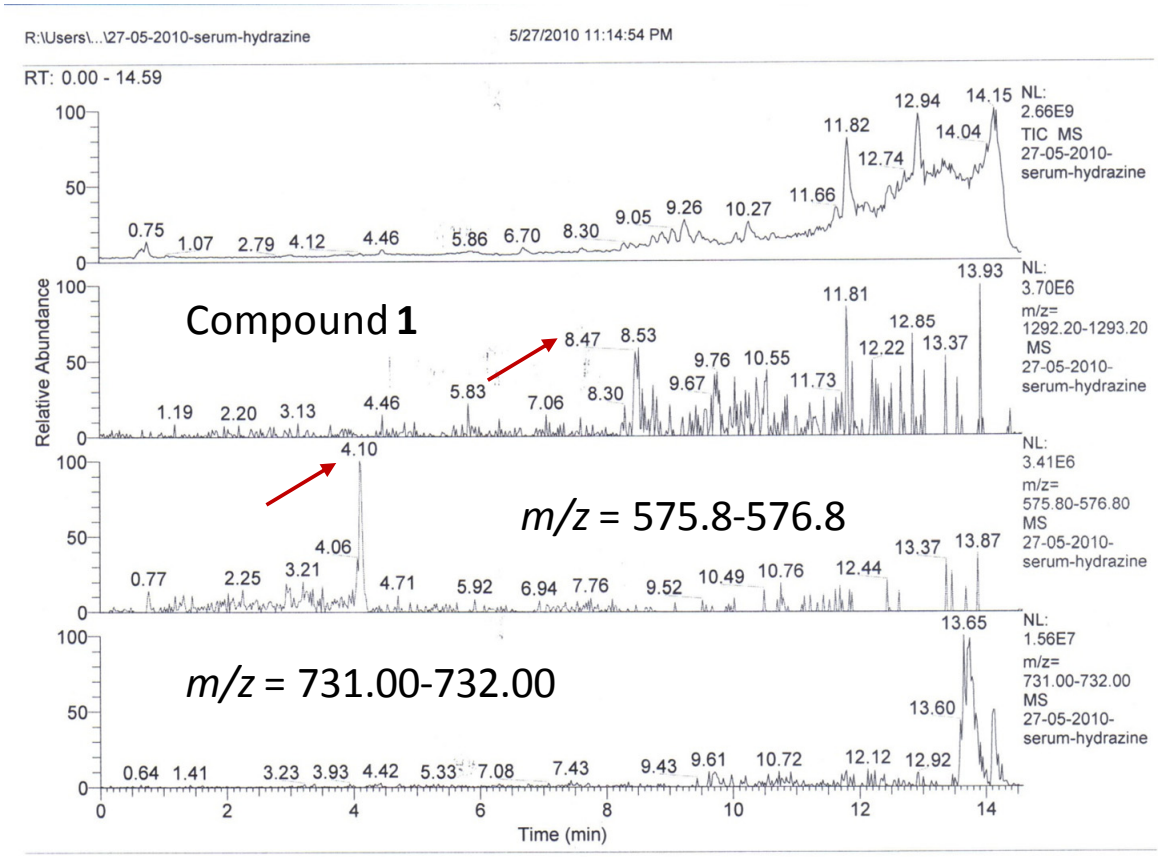


serum + compound **1**

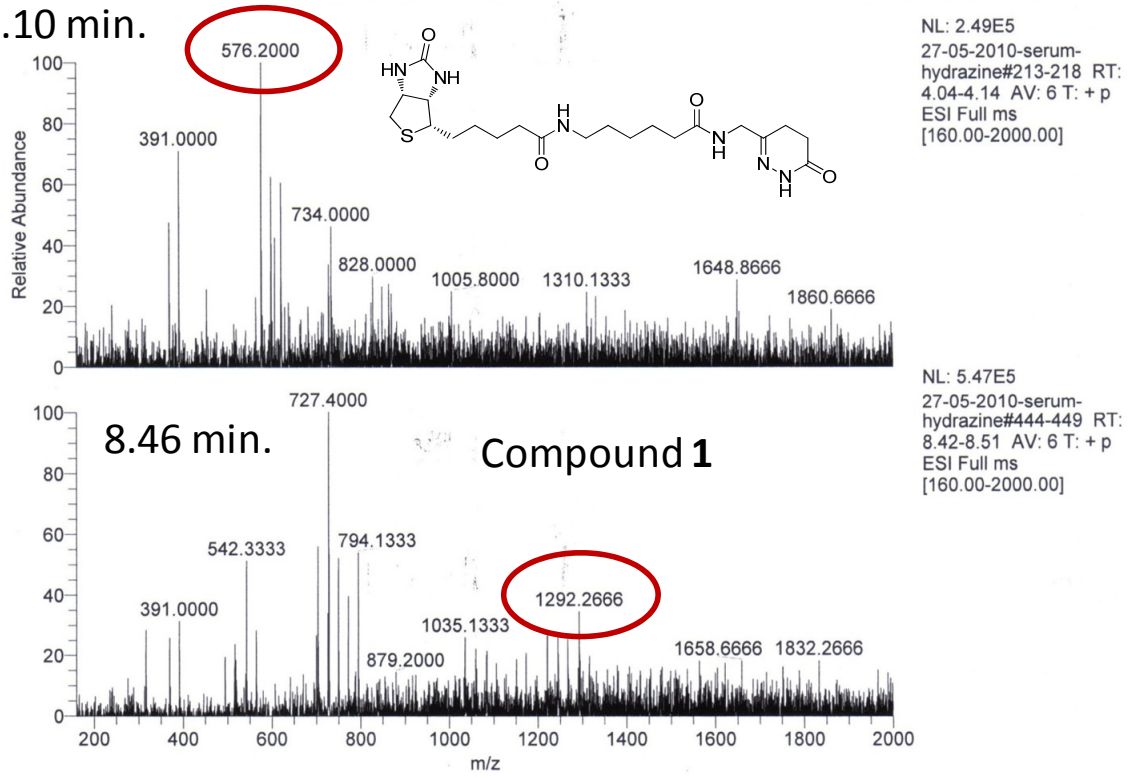




serum + compound **1** + H_2NNH_2



4.10 min.



References

[1] B. Roth, D. P. Baccanari, C. W. Sigel, J. P. Hubbell, J. Eaddy, J. C. Kao, M. E. Grace, B. S. Rauckman, *J. Med. Chem.* **1988**, *31*, 122–129.

[2] T. D. Fulmer, T. A. Bryson, *J. Org. Chem.* **1989**, *54*, 3496–3500.

[3] a) N. Sin, K. B. Kim, M. Elofsson, L. Meng, H. Auth, B. H. B. Kwok, C. M. Crews, *Bioorg. Med. Chem. Lett.* **1999**, *9*, 2283–2288. b) M. Verdoes, B. I. Florea, W. A. van der Linden, D. Renou, A. M. C. H. van den Nieuwendijk, G. A. van der Marel, H. S. Overkleeft, *Org. Biomol. Chem.* **2007**, 5, 1416–1426.

[4] K. M. Bonger, R. J. B. H. N. van den Berg, A. D. Knijnenburg, L. H. Heitman, A. P. IJzerman, J. Oosterom, C. M. Timmers, H. S. Overkleeft, G. A. van der Marel, *Bioorg. Med. Chem.* **2008**, *16*, 3744–3758.

[5] B. M. Kessler, D. Tortorella, M. Altun, A. F. Kisselev, E. Fiebiger, B. G. Hekking, H. L. Ploegh, H. S. Overkleeft, *Chem. Biol.* **2001**, *8*, 913–929.

[6] D. Wessel, U. I. Flügge, *Anal. Biochem.* **1984**, *138*, 141–143.

[7] A. Shevchenko, H. Tomas, J. Havlis, J. V. Olsen, M. Mann, *Nat. Protocols* **2006**, *1*, 2856–2860.

[8] J. V. Olsen, L. M. de Godoy, G. Li, B. Macek, P. Mortensen, R. Pesch, A. Makarov, O. Lange, S. Horning, M. Mann, *Mol. Cell. Proteomics* **2005**, *4*, 2010–2021.

[9] D. Baek, J. Villén, C. Shin, F. D. Camargo, S. P. Gygi, D. P. Bartel, *Nature* **2008**, *455*, 64–71.

[10] M. Verdoes, B. I. Florea, U. Hillaert, L. I. Willems, W. A. van der Linden, M. Sae-Heng, D. V. Filippov, A. F. Kisselev, G. A. van der Marel, H. S. Overkleeft, *ChemBioChem* **2008**, *9*, 1735–1738.